Thesis for Master of Pharmacy

Regulation of legumain activity by cystatin M in various cell lines

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Acknowledgments

Initially, I would like to express my deepest gratitude to my supervisors, Professor Rigmor Solberg and Professor Harald Thidemann Johansen for their excellent guidance and support throughout the year. I greatly appreciate their patience and quick responses during the writing process.

I am deeply grateful for all the assistance, generosity and practical guidance I have received from Hilde Nilsen during the laboratory period. I also would like to thank Mona Gaarder and Anne-Lise Rishovd at the ZEB-building for helping me with plasmid purification.

My thanks go to fellow master's candidates Sakina Ben Omar and Eilen Tungland for inspirational discussions and psychological support. I am also grateful to everyone at the fourth flour for their contribution to an enjoyable working environment.

I would like to take this opportunity to thank the closest people to my soul; my parents, brothers and sisters for their love and continuous encouragement.

Finally, very special thanks to my lovely wife Chiya for listening to my moaning and complaining and supporting me all the way. I thank her for creating a good working environment at home. Thanks to my little angle Honia for letting me enjoy uninterrupted night's sleep throughout the year.

Thanks!

Zana A. Ali

Oslo, May 2008

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Abbreviations

AEP	Asparaginyl endopeptidase
APS	Ammonium persulphate
AR	Androgen receptor
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
CHAPS	3- ((3-Cholamidopropyl)dimethylammonio)-1-propanesulfonic acid
CMV	Cytomegalo virus
CP	Cysteine protease
CPI	Cysteine protease inhibitor
dH_2O	Destillated water
DMEM	Dulbecco's Modified Eagles Medium
DTT	Dithiotreitol
DNA	Deoxyribonucleic acid
E64	Trans-epoxysuccinyl-L-leukylamido (4-guanidino) butan
FBS	Fetal bovine serum
ECL	Enhanced chemiluminescence
HBr	Hydrobromide acid
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HS	Horse serum
HSV	Herpes simplex virus
IU	Inhibitory units
kb	kilo bases
kDa	kilo Daltons
Ki	Inhibition constant
LAF	Laminar air flow

Leu-Leu-OMe	L-leucyl-L-leucine methyl ester
MHC	Major histo-compatability complex
mRNA	Messenger RNA
MMP	Matrix metalloproteinase
NH_2Mec	7-amino-4-metylcumarin
NSCLC	Non small cell lung cancer
OIP	Osteoclast inhibitory peptide
PA	Poly adenylation signal
PBS	Phosphate buffered saline
PDT	Photodynamic therapy
PMA	Phorbol-12-myristate-13-acetate
RNA	Ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RUH-HF	Rikshospitalet University Hospital HF
SDS	Sodium dodecylsulphate
SV	Simian virus
TAM	Tumour associated macrophage
TBE	Tris-borate EDTA
TCA	Trichloroacetic acid
TEMED	N,N,N,N-tetra-methyl-ethylenediamine
TGF-β	Tumour growth factor-β
Tris	Tris(hydroxymethyl)aminomethan
T-TBS	Tris-buffered saline containing 0.05 % Tween 20
TPI	Thiol-protease inhibitor
uPA	Urokinase plasminogen activator
VGEF	Vascular endothelial growth factor

Abstract

HEK 293 cells were transfected with LGMN-plasmid encoding rat legumain, to establish a cell line transiently and/or stably expressing legumain. In addition, HEK 293 cells and a number of melanoma cell lines (from Rikshospitalet University Hospital HF) were successfully transfected with CST6-plasmid encoding human cystatin M, a potent secreted endogenous inhibitor of legumain and other cysteine proteases. Successful CST6-transfection was evaluated as increase in total inhibitory activity (IU/ml) against papain as the target enzyme in media from transfected cells versus control cells (using empty vector; pTracer). The increase in total inhibitory activity was much higher in media from the CST6-transfected HEK 293 cells than the CST6-transfected melanoma cells. In addition, legumain activity was measured in cell lysates from CST6-transfected cells, showing decreased activity compared to control cells. The legumain activity was nearly abolished in cell lysates from CST6-transfected HEK 293 cells and moderately decreased (10-41 %) in CST6-transfected melanoma cells. Interestingly, decrease in legumain activity was strongly correlated to the potency of total inhibitory activity in media from the same cells. Despite many adjustments made to optimize transfection efficacy of HEK 293 cells with the LGMN-plasmid, legumain could not be over-expressed.

Cystatin M-conditioned media from CST6-transfected cells were used to treat living HEK 293, THP-1 and PC12 cells to investigate how this affected legumain activity in the cells. Legumain activity was only moderately decreased in cell lysates of HEK 293 cells treated with cystatin M-conditioned compared to control cell media. However, no regulation of legumain activity was observed in either THP-1 or PC12 cells after treatment with cystatin M-conditioned media.

Cell lysates from HEK 293, THP-1 and PC12 cells were subjected to size exclusion chromatography in an attempt to characterize the molecular weight of active legumain in these cells. Surprisingly, different molecular weights were found to be responsible for legumain activity in the different cell lines. Addition of cystatin M-conditioned media to HEK 293, THP-1 and PC12 cell fractions of highest legumain activity resulted in inhibition of legumain activity. Legumain activity in HEK 293 and PC12 cells was almost completely suppressed, but surprisingly only partially inhibited in THP-1 cells. Adding of the cathepsin-

inhibitors E64 and CA074 to the highest legumain activity fractions from the same cell lines resulted in no inhibition of legumain in HEK 293 and PC12 cell lysate fractions. On the contrary, the legumain activity was greatly decreased in the THP-1 cell fraction by both E64 and CA074. This is the first observation of legumain inhibition by E64 and CA074 in any cell type. This might indicate an uncharacterized form of legumain or a legumain-like enzyme in monocytes/macrophages.

1. Introduction

1.1 Proteases and cancer

Proteases (or peptidases) have been associated with many aspects of cancer pathophysiology [1-4]. Invasive tumour cells and their microenvironments are enriched with a number of proteases. Proteolysis plays a crucial role during the process of invasion and, consequently, metastasis of tumour cells via cleavage of important proteins involved in cell-cell adherence and extracellular matrix [2]. Additionally, protease activity facilitates activation of other proteases and the entrance of migrating tumour cells into circulation. Thus by further degradation of extracellular matrix components, proteases stimulate tumour growth, invasion and angiogenesis through liberation of embedded growth factors in the surrounding matrix [2]. In support of previously mentioned findings, an inhibitor of matrix metalloproteinase (MMP), Batimastat®, has been shown to cause decreased migration and invasion of tumours when applied to a number of tumour cell lines [5]. In addition, examining low molecular weight inhibitors of MMP and urokinase plasminogen activator (uPA) led to the conclusion that inhibiting proteases had a tumour suppressing and anti-metastatic effect [6].

1.1.1 Cysteine proteases

Proteases are enzymes that catalyze breakdown of proteins by cleaving peptide bonds upon nucleophilic attack on the carbonyl bond [7]. The cleavage of protein substrates takes place either from the N- or C- terminal end of the protein (exopeptidases), and/or in the middle of the target protein (endopeptidases). In general, five major types of proteases have been identified (cysteine, serine, threonine, aspartate, and metalloproteinases) [8]. These names refer to the catalytic site of the enzyme. Cysteine endopeptidases or cysteine proteases (CPs) represent one of the major groups of proteolytic enzymes. These can be divided into about 30 separate families based on their molecular structure. In mammals, three families of cysteine proteases were studied thoroughly (C1, C2, C14) before identification of legumain family (C13) as the fourth member of cysteine endopeptidases in mammals [9]. The majority of proteases are synthesized as inactive precursors being activated by proteolytic removal of the N-terminal propeptide. Removal of the propeptide can be facilitated either by the action of other proteases, or by autoactivation [10, 11].

The papain family (C1) is the most numerous in mammals and includes cathepsin B, H, L, S and others. The human genome is now known to contain 11 related cathepsins: B, F, H, K, L, O, S, V, X, dipeptidyl-peptidase I (cathepsin C) and cathepsin W [8, 12]. These lysosomal proteases are responsible for proteolysis intra-lysosomally/endosomally, but can also be secreted to act out side the cells [13]. The function of the cathepsins B, H, and L is degradation of intracellular proteins. Cathepsins K and S are implicated in the remodelling of bone and antigen presentation, respectively [14]. Despite their crucial functions in the body, the cathepsins have been found to be implicated in a number of diseases such as osteoporosis, rheumatoid arthritis, osteoarthritis and cancer, as well as in immune response and neurodegeneration [13].

The calpain (C2) and the caspase (C14) families are located intracellularly in the cytosol [9]. Although functions of these two families of cysteine proteases have not been fully clarified, some of the caspases have been reported to play important roles in inflammation and apoptosis [8, 15]. The more recently discovered legumain family (C13) is the latest member of the family of cysteine proteases to date. The biological functions of this family of cysteine endopeptidases and its involvement in tumours, as well, will be discussed in detail later.

1.1.2 Cysteine proteases and cancer

A number of studies have been presented supporting that cysteine cathepsins contribute to tumour progression [2, 3]. In certain tumours, cysteine cathepsins are shown to become translocated from their intracellular compartments to the cell surface [1, 2]. Reports have revealed that several cathepsins were up-regulated during tumour progression, and they contributed to the capability for invasive tumour growth. In addition, cathepsins were shown to enhance angiogenic switching, tumour vascularity, invasion, proliferation of tumours, and to be involved in apoptosis [2-4, 16]. Calpains have also been described to be involved in cancer progression through a number of studies [7].

The function of caspases as apoptotic initiators and executioners is a well known fact and has been demonstrated in a number of studies [17-21]. Caspase-1 has recently been shown to be down-regulated in the progression of ovarian and gastric cancers. On the other hand, intra-prostatic administration of inducible caspase-1 and interleukin-12-containing adenoviruses caused local cell death and improvement of survival of adenocarcinoma-bearing mice [7].

1.1.3 Cysteine protease inhibitors

What have made protease inhibitors of considerable interest as drug candidates is observations that have connected tumour suppressing properties to cysteine protease inhibitors (CPIs) [5, 22-25]. Improper proteolytic activity has long been known to have prominent role in cancer as well as cardiovascular, inflammatory, neurodegenerative, bacterial, viral and parasitic diseases. Therefore, developing protease inhibitors is one of the most popular fields of drug discovery by pharmaceutical companies [26]. In normal tissues, the activity of proteases is tightly controlled in order to inhibit improper cleavage of signalling proteins [26].

During the last four decades, considerable amounts of effort have been made on developing inhibitors against cysteine proteases. In 1960, reports presented evidence for factors capable of inhibiting clotting activity of a thiol-dependent protease [27]. Furthermore, other groups described that cysteine (or thiol) protease inhibitors (CPIs or TPIs) were able to inhibit the plant cysteine proteases ficin and papain [27]. Further exploration of CPIs revealed that they belonged to a protein super-family, the cystatin super-family, which inhibited papain-type cysteine proteases [28].

Interestingly, a recently published review listed more than ten cysteine protease inhibitors at different phases of clinical developments [15]. Among these novel inhibitors, the pan-caspase inhibitor IDN-6556 has newly gained approval from the Food and Drug Administration (FDA) in USA to treat patients undergoing liver transplantation particularly, and other solid organ transplantations as well. Recently, several epoxysuccinyl-based inhibitors have been reported to have selective and potent inhibitory effects against papain-like cysteine proteases, both *in vivo* and *in vitro* [29]. The epoxysuccinyl peptide derivatives, E64 and its synthetic analogues, have acquired most attention among the many natural cysteine protease inhibitors that have been isolated from dry-land microorganisms [15]. Besides, other natural inhibitors against cathepsin B, in addition to epoxysuccinyl peptides, have been identified; peptidyl aldehydes and aziridinyl peptides [30]. Newly, a number of cathepsin B inhibitors were disclosed, including bis-peptidyl inhibitors, cyclohexanon-based inhibitors, a series of azapeptides possessing an azaglycine residue and dipeptidyl nitrile [15 62]. Dipeptidic nitriles are described as potent and selective cathepsin B inhibitors compared to the other inhibitors mentioned above [15, 31]. In addition, inhibitors of cathepsins K and S are, as well, in various phases of clinical development [15]. Similarly, a number of

compounds are being explored and developed as in-activators of calpain (C2) and caspases (C14) in connection with different types of diseases. Chagasin, the inhibitor family I42 (*MEROPS*: the peptidase database) of cysteine proteases from protozoan parasites as well as prokaryotes and eukaryotes, was discovered. It was found to be tightly bound to the human cathepsins (B, H, K, and L). Although chagasin and the cystatins have different sequence homology, they were found to have remarkably similar binding interactions [32].

1.2 Legumain

Legumain (asparaginyl endopeptidase, AEP) is a novel lysosomal protease belonging to clan CD. It is a member of the (C13) family of cysteine proteases, whereas all other known lysosomal cysteine proteases belong to C1 family (papains). Legumain has a highly restricted specificity, since it cleaves only substrates having an asparagine residue at P1 position [9]. Under acidic environments, however, legumain can also cleave bonds after aspartate [11]. It was first discovered by Csoma & Polgar in 1984 in germinating bean cotyledons, and was later found in several other plant seeds and tissues [33]. Moreover, the enzyme has also been described in the parasite blood fluke *Schistosoma mansoni* [34]. Cloning and sequencing of mammalian legumain cDNA, as well as isolation and characterization of the enzyme from pig kidney were done by Chen and co-workers in 1997 [9]. The cDNA of human legumain encodes a prepro-protein of 433 amino acid residues (49 kDa) [33].

By investigating the catalytic site of legumain (histidine and cysteine), it has been proposed that it has similar protein folds, and thus an evolutional relationship to the caspases, gingipains and clostripain [35]. Therefore, these enzymes are placed together in the clan CD.

In recent years, many reports have associated legumain to a number of interesting and considerable functions. The enzyme has been described to play a prominent role in the processing of bacterial antigens by MHC II [36]. Legumain has also been reported to activate the zymogen progelatinase A (pro-MMP2) to gelatinase A (MMP2), which plays an important role in degradation of extracellular matrix [37]. Furthermore, data has been presented for a vital role of legumain in remodelling of extracellular matrix of renal proximal tubular cells via degradation of fibronectin, one of the main components of extracellular matrix, both *in vivo* and *in vitro* [38]. To support this result, it was shown that fibronectin processing could be inhibited by co-incubation of chloroquine, an inhibitor of lysosomal degradation. Moreover, legumain has been identified to inhibit formation of osteoclasts and

bone resorption by more than 60 % and it has, consequently, been termed osteoclast inhibitory peptide-2 (OIP-2) [39, 40]. It has also been described to be involved in the cornification of skin where CST6 deficient mice (not expressing cystatin M/E) showed abnormal skin cornification [41].

Legumain is not ubiquitously expressed in human tissues, but it has been shown to present in tissues from kidney, liver, spleen, placenta and heart [42]. In an attempt to localize legumain subcellularly in mouse tissues, it was visualized to be primarily located in lysosomes [43]. Very recently, legumain activity and expression were found to be dramatically increased when THP-1 cells (human monocytic cell line) were differentiated towards macrophages upon activation by PMA (phorbol 12 myristate 13-acetate) [44].

1.2.1 Legumain processing and activation

For optimal catalytic activity and stability, legumain requires an acidic environment [9, 45]. The acidic "window" for optimal legumain autoactivity has been described to be the pH range 3.5-4.5 [46] (figure 1).

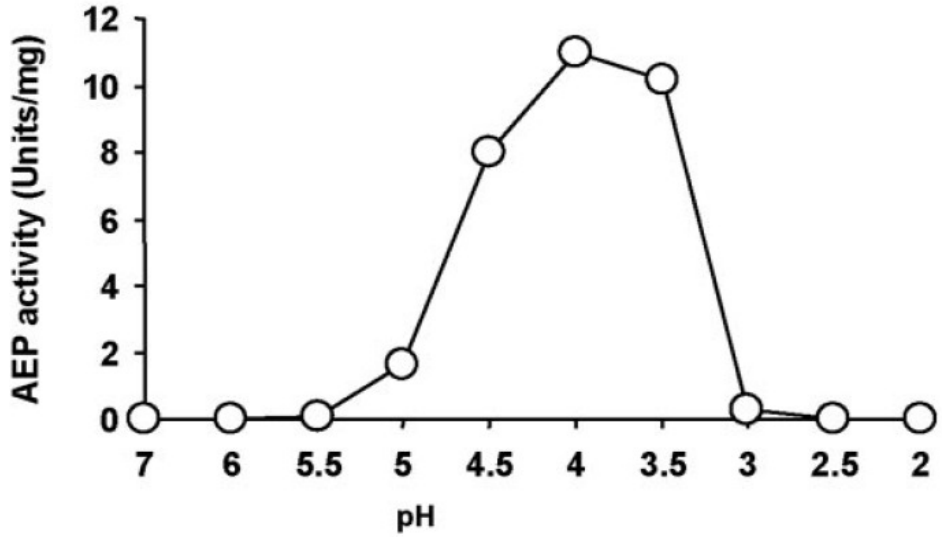

Figure 1: Autoactivation of legumain (AEP) (Li et al. 2003). *Purified precursor (56 kDa) pig kidney legumain was incubated at the pH values indicated for 2 h at 37 °C. Catalytic activity of legumain in each aliquot of the reactions (at different pHs) was measured using the substrate Z-Ala-Ala-Asn-NHMec.*

In order to be activated, legumain has to go through two successive cleavages of C-and N-terminal prepro-proteins, respectively, at different pH thresholds. Sequential cleavage after both asparagine and aspartic acid were found to be necessary for efficient enzyme activation resulting in the removal of a 110-residue C-terminal and 8-residue N-terminal propeptide,

respectively [46]. Like other lysosomal proteases, legumain is translated as preproform, transferred through the Golgi apparatus as the proform (56 kDa), and localized in late endocytotic compartments (lysosomes) as the mature enzyme (46 kDa) [47]. To support the fact that activation of prolegumain to active legumain is autocatalytic requiring acidic conditions [11, 46], one research group reported that legumain processing was inhibited when ovocystatin (egg-white cystatin), an inhibitor of legumain, was added to the cell lysates from stable transfectants of legumain cDNA [47]. To investigate processing of the proform of legumain *in vivo*, transfected HEK 293 cells with LGMN-cDNA were cultured in growth media containing different protease inhibitors. The processing protease for legumain was anticipated to be a papain-type cysteine protease, since only E64, not ovocystatin, inhibited legumain processing *in vivo* [47]. Later on, Li and co-workers [46] concluded that the final step in activating the mature legumain (46 kDa) is not autocatalytic, but occurs as a result of normal cellular processing involving other lysosomal proteases, since no processing beyond the 46 kDa product was obtained *in vitro*. Additionally, they documented that legumain found in living cells is a 36 kDa peptide, which is smaller than the autocatalytically activated 46 kDa (figure 2).

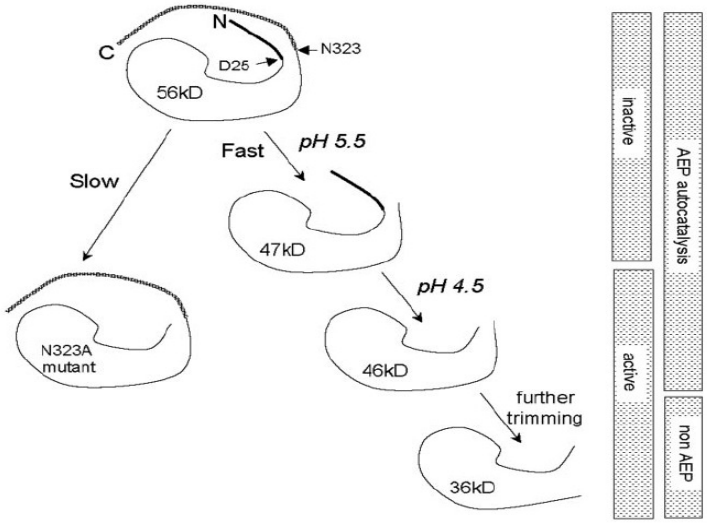

Figure 2: Cellular processing of legumain (AEP) adapted from Li et al. 2003 [46]. *N323A mutant is a mutant form of AEP which is activated much more slowly than the wild type AEP at the same pH.*

1.2.2 Legumain in cancer

As mentioned earlier, many proteases are up-regulated and involved in tumour progression, enhancing angiogenesis and invasion, and involved in degradation of extracellular matrix

proteins [2-4, 16]. Legumain has been documented to activate pro-MMP2, and hence increase degradation of extracellular matrix [37]. Evidence has been presented for overexpression of legumain in a number of solid tumours (i.e. breast carcinoma, colon carcinoma, lung carcinoma, prostate carcinoma, ovarian carcinoma, central nervous system tumours, lymphoma and melanoma) [42]. The expression has been demonstrated to be highest in prostate tumours. Notably, powerful correlation between its overexpression and enhancement of migratory and invasive properties *in vitro* and *in vivo* was documented. HEK 293 cells overexpressing legumain were found to migrate and be more invasive than control HEK 293 cells. Moreover, similar findings were evident *in vivo* when mice were injected with control 293 cell tumour or legumain+293 cell tumour [42]. Interestingly, increased migration and invasion *in vitro* were partially inhibited by cystatin, a potent cysteine protease inhibitor. Although primarily in lysosomes, legumain has also been reported to appear extracellularly in the acidic tumour microenvironment, associated with matrix as well as cell surfaces [48]. In addition, legumain was found to be expressed by tumour angiogenic endothelial cells.

Remarkably, legumain has been shown to activate the cell-impermeable prodrug legubicin which was synthesized by incorporating a peptide extension to the amino group of doxorubicin [42, 48]. Legubicin exhibited reduced toxicity relative to doxorubicin, and was found to be effectively tumoricidal *in vivo* without inducing any injury to other tissues normally expressing legumain (i.e. kidney).

Recently, legumain was reported to be overexpressed in tumour associated macrophages (TAMs). TAMs contribute to promotion and proliferation of tumour cells by secreting growth factors and proangiogenic factors as well as metalloproteinases [49]. Notably, a full-length murine legumain-based DNA vaccine showed increase in cytotoxic T-cell response against TAMs and hence reduction in TAM density. This in turn, led to suppression in tumour angiogenesis and marked improvement in survival of murine models of different tumour types [49]. Very recently, the same research group generated an oral legumain based minigene vaccine against TAMs which targeted tumour stroma [50]. This minigene vaccine induced immune responses directed against specific antigen epitopes while avoided responses against irrelevant antigen epitopes which may cause serious side effects. Administration of the legumain-based minigene vaccine to murine breast tumour models resulted in a marked suppression of tumour growth, metastasis, and angiogenesis. In support

to the above, in a study performed on colorectal cancer materials, it was concluded that legumain was overexpressed and had a prognostic value [51].

In a newly published study, legumain expression and its prognostic value were investigated in invasive breast cancer and non-cancerous breast tissues by immunohistochemistry [52]. It was observed that vesicular expression of legumain was dominant in 24 % of carcinomas and was associated with a more adverse outcome. These observations were in agreement with findings reported in previous work which suggested that overexpression of legumain was a prognostic factor and was correlated to more invasive and highly metastatic tumours [42, 51].

1.2.3 Inhibitors of legumain

The clan CA enzymes (papain, calpains and cathepsins) are inhibited by the epoxide inhibitor E64 and use a catalytic triad in their active side. Clan CD cysteine proteases (legumains, caspases, gingipains, clostipains, and separase) are, on the contrary, resistant to inhibition by E64 and use a catalytic dyad in their active site [53]. In the literature, there has been described a handful of legumain inhibitors [53-57]. Cysteine protease inhibitors, the cystatins, are the most potent inhibitors of legumain described to date [23, 27, 43, 56, 57]. Cystatins M/E and C have been reported to be the most potent inhibitors of legumain among the members of the cystatin super family [56, 57]. In addition, other inhibitors of legumain have also been reported [9, 53-55]. When legumain was identified in mammals, Chen and co-workers tested a number of potential inhibitors of pig legumain [9]. Whereas general inhibitors of serine, aspartic and metalloproteases were not able to inhibit legumain, *p*-chloromecuribenzoate, iodoacetate, iodoacetamide, and *N*-ethylmaleimide were reasonably effective inhibitors. Ovocystatin (from chicken egg-white) and recombinant human cystatin C, on the other hand, were slow, tight-binding inhibitors of legumain. A series of Michael acceptors (from the Michael reaction; α,β-unsaturated carbonyl compounds) based on the backbone Cbz-L-Ala-L-Ala-L-Asn (Cbz: benzyloxycarbonyl) have been demonstrated to be potent irreversible inhibitors of legumain. Among these; Cbz-L-Ala-L-Ala-L-Asn-CMK (CMK: Chloromethylketone) was shown to be the most potent inhibitor of pig legumain [54]. Subsequently, caspase specific inhibitors were shown to be capable of inhibiting legumain as well [55]. Further, new acylmethylketone inhibitors of legumain have been described, showing that 2, 6-dimethyl-benzoic acid 3-benzyloxycarbonylamino-4-carbomoyl-2-oxo-butyl ester (MV026630) was the most potent [58]. Aza-peptide epoxides have also

been described as potent and selective inhibitors of legumain from *Schistosoma manosi* and pig kidney, and these showed little or no inhibition against other clan CA or CD proteases [53].

1.3 Cystatins

Cystatins are potent endogenous inhibitors of lysosomal cysteine proteases [28, 56]. The first member of this superfamily of enzyme inhibitors, ovocystatin, was described in the late 1960s [14]. All functional cystatins are inhibitors of cysteine proteases of the papain (C1) family, and some also inhibit enzymes in the legumain (C13) family [14]. Cystatins regulate normal body processes through regulation of the activity of lysosomal cysteine proteases which can cause diseases when over-expressed, unless their activity is firmly controlled by inhibitors.

There are three major families or types of cystatins; type 1 cystatins (A and B), type 2 cystatins (C, D, M/E, F, G, S, SN, and SA), and type 3 cystatins (L- and H-kininogens). However, plant cystatins (phytocystatins) are also described as another type or family of cystatins. Type 1 cystatins (also called stefins) are mainly located intracellularly, whereas type 2 cystatins are secreted and located extracellularly, and type 3 cystatins intravascularly. Very recently, it was reported that carboxy terminal extended phytocystatins are bifunctional inhibitors of papain and legumain [59]. By investigating a panel of cystatin super family members, it was demonstrated that type 1 (stefins A and B) and (type 3) cystatins were not inhibitors of pig legumain. However, type 2 cystatins F and D were shown to be inhibitory and non-inhibitory of pig legumain, respectively [56]. On the other hand, type 2 cystatins C and M/E has outstandingly good inhibitory properties for all papain-like proteases investigated, as well as legumain [14].

The type 1 cystatins is a large group of small proteins, approximately 11 kDa in size, which lack disulfide bonds. Members of the type 2 cystatins are small proteins of approximately 12–13 kDa containing two disulfide bonds towards their C-terminus. Type 3 cystatins (the kininogens) are large acidic proteins (45–65 kDa) containing one or several cystatin domains [14, 60].

1.3.1 Human cystatins and their roles in cancer

It has been suggested that the change in the balance between cysteine proteases and their inhibitors (cystatins) could alter the susceptibility to cancer and tumourigenisity [8]. Many papers have reported that cystatins are strongly implicated in cancer [8, 22-25, 27, 42]. Below, some of the most important cystatins and their implication in cancer will be discussed:

1.3.2 Stefin A

A growing amount of evidence is suggesting that stefins A and B regulate initiation or propagation of the lysosomal cell death pathway [27]. The expression of stefin A has been found to be lost during progression of most prostate and breast cancers, as well as during tumourigenesis of skin, metastasis in oral carcinoma and lung cancer progression. Interestingly, exogenous stefin A was shown to inhibit motility of melanoma cells without affecting viability of the cells [27]. Recently, it was reported that levels of stefins A and B as well as the activity of cysteine proteases were significantly greater in small cell lung tumours when compared to normal lung tissues. Moreover, the survival probability of the patients was found to be better when levels of stefins A and B, and cysteine protease activity were elevated [24].

1.3.3 Stefin B

Stefin B is a far more ubiquitous protein than stefin A, but its level of expression is varying between different tissues [27]. In tumour cells, it has been shown variation in stefin B levels when compared to normal non-tumour cells. Similar to exogenous stefin A, exogenous stefin B has been found to inhibit the motility of melanoma cells [27].

1.3.4 Cystatin C

Cystatin C is a secreted protein found in all tissues and body fluids. Cystatin C is the most thoroughly studied cystatin in mammals. Mature human cystatin C is composed of 120 amino acid residues and is synthesized as a preprotein [14]. The concentration of cystatin C in normal human serum is about 77 nM (1.16 µg/ml). Due to low molecular weight (15 kDa), cystatin C is efficiently eliminated via glomerular filtration. Consequently and due to other features, cystatin C is an excellent marker of the glomerular filtration rate. Expression of cystatin C in premalignant and malignant cells does not appear to alter much. On the contrary, some reports have shown elevated levels of cystatin C in sera, pleural effusions and

ascitic fluids gathered from cancer patients. The clinical value of these high levels of cystatin C in cancer patients, however, is yet to be clarified. In a study, it has been observed increased levels of cathepsin B and decreased level of cathepsin B/cystatin C complexes in lung cancer patients versus patients with non-cancerous lung disease or healthy individuals [27]. Cystatin C is assumed to be accumulated in pleural effusions rather than in the serum of cancer patients, it is therefore unable to inactivate serum cathepsin B. Cystatin C have been shown to inhibit *in vitro* tumour cell mediated degradation and invasion of extracellular matrix [27]. Cystatin C has been described as tumour growth factor-β (TGF-β) receptor antagonist and thus mediating effects on cellular signalling. Potent anti-cancer properties were obtained when cystatin C was studied in human SNB-19 glioblastoma cells and in a mouse model of glioblastoma. The tissue levels of cystatins C and M/E were obtained to be down-regulated in non small cell lung cancer (NSCLC) tumours when compared to healthy lung tissue [24]. These lower levels of type 2 cystatins, however, provided no prognostic information. In a current attempt to explore the underlying molecular mechanism in prostate cancer *in vitro*, cystatin C was reported to inhibit invasion of cancer cells in cooperation with TGF-β receptor II and androgen receptor (AR) pathways [61]. In a recent paper, photodynamic therapy (PDT) in combination with ovocystatin, have been documented to cause extensive tumour necrosis and significant decrease in serum vascular endothelial growth factor (VGEF) levels when compared to cystatin and PDT alone in a rat mammary tumour model [62].

1.3.5 Cystatin M/E

Cystatin M/E is a low molecular mass protein that is secreted in both a glycosylated (17 kDa) and an unglycosylated (14 kDa) form. Cystatin M, and other cystatins, have been reported to be strongly involved in cancer [8, 22-25, 27, 42]. Ten of 12 established human breast cancer cell lines were shown to lack expression of cystatin M, while normal and premalignant cells expressed plentiful levels of the transcript. Additionally, *scid* (severe combined immunodeficient) mice orthotopically implanted with breast cancer cells expressing cystatin M, have been shown to considerably delay primary tumour growth and lower metastatic rate in lungs and liver when compared with mice implanted with mock controls (not expressing cystatin M) [25]. Based on these observations, it was suggested that the lack of cystatin M is contributed to the progression of human breast cancer.

Highly tumourigenic and metastatic breast cancer cell lines, MDA-MB-435S, were stably transfected with a cystatin M expression vector and studied for the outcomes of this expression on the malignant properties of the cells [22]. It was found that cystatin M expression reduced cell proliferation, migration, matrix invasion and tumour-endothelial cell adhesion. Notably, cell migration and matrix invasion appeared to be dependent on cysteine proteases, as both recombinant cystatin M and E64 blocked such processes. In support of these determinations, the activity of legumain and cathepsins (B and L) as well as cell proliferation and *in vitro* invasion were shown to be increased in metastatic oral cancer cell lines when cystatin M was silenced by siRNA [23]. As a consequence, it is assumed that cystatin M may have the ability to regulate the intracellular activities of their target cysteine proteases, beside their functions as secreted inhibitors of cysteine proteases.

Very recently, the gene for cystatin M was reported to be frequently methylated, and hence inactivated in a number of breast cancer cell lines when compared to normal breast-tissue samples [63]. This hypermethylation of the cystatin M-promoter was shown to lead to inhibition of its transcription and silencing of cystatin M. Additionally, other reports have shown considerable reduction or loss of cystatin M expression in a number of skin cancer cell lines [27].

1.3.6 Cystatin F

Cystatin F (also known as leukocystatin or CMAP) is also a secreted form of the type 2 cystatins, composed of 126 amino acid residues [14] being predominantly expressed in hematopoietic cells [27]. The gene for cystatin F has been reported to be highly expressed in a number of metastatic human cancer cell lines, and has been contributed to a higher rate of liver metastasis [27]. Suppressing this gene has, interestingly, shown reduction in metastasis of the tumour cells to liver and spleen, and improved survival of tumour-bearing mice. Cystatin F acts in contrast to, and in a completely different manner from cystatins C and M by stimulating rather than suppressing metastasis to the liver. In addition, a 5-years survival study confirmed the correlation of higher levels of cystatin F to a significantly worse survival rate of the patients [27].

2. Aims

Legumain has been shown to be a possible contributor to malignancy of cancer cells, and has been suggested as a marker for tumour invasion and metastasis. Contrary, cystatin M is the most potent endogenous inhibitor of legumain and has been suggested to be a tumour suppressor candidate.

There might be a possible relationship between uncontrolled proteolysis by legumain and important regulatory and protective functions by cystatin M.

This study wants to study the interplay between these proteins by:

- Establishing methods in our laboratory for over-expressions of legumain and cystatin M.
- Determining whether over-expression of cystatin M regulates the activity of legumain in the same cell.
- Determining whether exogenously added cystatin M regulates intracellular legumain activity.

Additionally, other objectives of this study are:

- Characterization of molecular weight of active legumain in different cells and correlation with cathepsin B.
- Studying the inhibitory profile of legumain in different cells using various cysteine protease inhibitors.

3. Materials

3.1 Cell lines

The following cell lines were used in this study:

- A human embryonic kidney cell line (HEK 293 cells) (American Type Culture Collection, CRL-1573):

  HEK 293 cells are adherent cells at 37 ^{o}C, and loosen at room temperature. These cells grow well both in medium with or without serum [64].

- A human acute monocytic leukaemia cell line (THP-1 cells) (American Type Culture Collection, TIB-202):

  THP-1 cells are a monocyte-like cell line isolated from a one year old boy with acute lymphatic leukemia. THP-1 cells are not attached to plastic under normal conditions. These cells become adherent upon stimulation with phorbol-12-myristate-13-acetate (PMA) (40 mg/ml) and they differentiate to macrophage-like cells [44].

- A rat pheochromocytoma cell line (PC12 cells) (American Type Culture Collection, CRL-1721):

  PC12 cells are derived from a pheochromocytoma (tumour of adrenal gland producing catecholamines) of the rat adrenal medulla. These cells form loosely adherent, multicell aggregates which loosen by tapping the flask.

- Various human melanoma cell lines (Rikshospitalet University Hospital HF; RUH-HF, table 1).

Table 1: Human CST6-transfected melanoma cell lines, received from RUH-HF.

Established melanoma cell lines	**Malignant melanoma cell lines from patients**
SKMEL-28 FEMX-I HHMS A-375	MM 11 MM 35 MM 69b

3.2 Chemicals and reagents

2-mercapto ethanol	Sigma-Aldrich, St. Louis, USA
Acetic acid (100 %)	Gibco, Life tech., Invitrogen AS, Norway
Acrylamide/Bis solution (37.5:1)	

(Monomer solution)	BioRad, Hercules, USA
Agarose	MedProbe, Oslo, Norway
Albumin	Pierce, Boule Nordic AS
Ammonium persulphate	BioRad, Richmond, CA, USA
Brij 35	Sigma-Aldrich
Bromophenol blue	Sigma-Aldrich
CA074	Calbiochem (Merck, Darmstadt, Germany)
$C_6H_5Na_3O_7.2H_2O$	Merck
$CaCl_2.2H_2O$	Merck
CHAPS	Sigma-Aldrich
Dye reagent concentrate (protein assay)	Bio-Rad
DL-Dithiotreitol (DTT)	Sigma-Aldrich
DMEM (HEK 293)	Invitrogen, art. No. 41966-052
DMEM (PC12)	Gibco, art. No.42430-025
ECL	GE Healthcare/ Amersham, England
E64	Sigma-Aldrich
Ethanol	Arcus, Oslo, Norway
Ethidium bromide (1 %)	Sigma-Aldrich
Non-fat dry milk	Normilk, Stavanger, Norway
Fungizone	Bristol-Meyers Squibb, NY, USA
FBS	Gibco
Glucose	Sigma-Aldrich
Glycine	VWR international, England
HBr	Sigma-Aldrich
HEPES	Sigma-Aldrich
HS	Invitrogen
Isobutanol	Merck
KCl	Prolabo, Paris, France
KHP_2PO_4	Prolabo
Lipofectamine 2000	Invitrogen
Methanol	Merck
$MgSO_4.7H_2O$	Prolabo

$Na_3C_6H_5O_7.2H_2O$ (sodium citrate)	Merck
NaCl	Prolabo
Na_2EDTA	Chemi-Technique, Berlin, Germany
Na_2HPO_4	Merck
$Na_2HPO_4.H_2O$	Merck
n-octyl-β-D-glucopyranoside	Sigma-Aldrich
Papain	Sigma-Aldrich
Penicillin-Streptomycin	Sigma-Aldrich
PMA	Sigma-Aldrich
Ponceau S solution	Sigma-Aldrich
Precision Plus Protein Standard	BioRad
RPMI 1640 with L-Glutamin	PAA Laboratories GmbH, Austria
SDS	BioRad
Sodium pyrovate	Gibco
TCA	Merck
TEMED	BioRad
Tris (base)	Sigma-Aldrich
Tryptan blue	Sigma-Aldrich
Tween 20	BioRad
X-ray developer LX24	Kodak, Oslo, Norway
X-ray fixer AL 4	Kodak
Z-Ala-Ala-Asn-NHMec	Department of Biochemistry, University of Cambridge, UK
Z-Arg-Arg-NHMec	BACHEM, Bubendorf, Switzerland
Z-phenyl-arginin-NHMec	BACHEM

4. Methods

4.1 Cell culturing and harvesting

Three different cell lines were cultured and used in the experiments: HEK 293, THP-1 and PC12 cells. All work with cells was carried out in a LAF bench using aseptic techniques. For cell culturing, a 75 cm^2 Corning cell culturing flask was used, while for experiments, six-well plates (Corning) were used. The cells were incubated at 37 ^oC in a humidified 5 % CO_2 atmosphere.

4.1.1 HEK 293 cells

HEK 293 cell culturing was performed in a Dulbecco's Modified Eagles Medium (DMEM) with high glucose content (appendix). The growth medium was changed every third/fourth day. Frozen cells (in liquid nitrogen tank) were thawed at 37 ^oC and cultured in 10-12 ml growth medium in a cell culturing flask. The cells were split whenever 80-90 % confluent, usually every fourth day. Trypsin-EDTA (0.5 % in serum-free DMEM) was added to detach the cells, and subsequently deactivated by adding growth medium containing 10 % serum. The cells were centrifuged (800 rpm, 5 minutes) and the pellet was resuspended in the growth medium. Finally, $1x10^6$ cells were transferred to a flask containing 10-12 ml growth medium (appendix). For experiments, cells were counted as described in 4.1.4.

4.1.2 THP-1 cells

Frozen THP-1 cells were thawed at 37 ^oC and cultured in 10 ml growth medium (appendix). The cells were split whenever the concentration of the cells was $1x10^6$ cells/ml, usually once weekly. The cells were centrifuged (800 rpm, 5 minutes), and the pellet was resuspended in growth medium followed by transferring $1x10^6$ cells to a cell culturing flask containing up to 10 ml growth medium. The growth medium was refreshed every third day by adding 10 ml fresh medium. Cell counting was performed as described in 4.1.4. For experiments, THP-1 cells were PMA stimulated (40 ng/ml) for 24 hours and washed with RPMI without additives (appendix).

4.1.3 PC12 cells

Frozen PC12 cells were thawed at 37 ^oC and cultured in 11 ml DMEM with high glucose content (appendix). Cells were split whenever 80-90 % confluent (approximately every

third/fourth day). Old medium was aspirated and the flask was hit to dislodge the cells. Fresh medium was added to break clusters by pipetting. One ml of this suspension was added to a culturing flask containing 11 ml fresh medium and the cells were incubated at 37 ^{o}C and 5 % CO_2. The medium was renewed every 2-3 days. For experiments, the cells were counted as described in 4.1.4.

4.1.4 Cell counting

In order to seed the cells (HEK 293, THP-1 and PC12) for experiments, the cells were counted by using a Bürker counting chamber. The cell suspension (100 µl) was added to 100 µl 0.4 % sterile filtered tryptan blue (appendix) in an eppendorf tube, and applied to the chamber. The cells in the diagonals were counted under microscopy and quantified using the following equation:

n (average of number of the cells in diagonals) x 4.2/100 = Cells x 10^6 per ml.

4.1.5 Cell harvesting

To harvest the cells (HEK 293, THP-1 and PC12), medium was aspirated and the cells were washed 1-3 times with 0.5-1 ml of PBS (for HEK 293 cells, 0.5 ml pre-warmed PBS was used because cells could detach) before adding 0.5 ml lysis buffer (appendix) to each well of six-well plates. In experiments for total inhibitory analysis, the media were collected as well. The cell lysates were transferred to eppendorf tubes and exposed to three cycles of freezing and thawing (-70 and +30 ^{o}C, respectively). Afterwards, the samples were centrifuged at 10000 g, and 4 ^{o}C for 5 minutes. The media samples were centrifuged as well and the supernatants were transferred to new eppendorf tubes. When un-stimulated THP-1 cells were harvested, the cells were first centrifuged (800 rpm for 5 minutes) and subsequently the media were aspirated before the cells were washed in PBS and lysed in lysis buffer as described above.

4.2 Amplification, purification and verification of plasmids

The LGMN-plasmid coding for rat legumain (rLGMN) was kindly provided by Professor Kazumi Ishidoh, Division of Molecular Biology, Institute for Health Sciences, Tokushima Bunri University, Japan. This was prepared by inserting the full length rLGMN-cDNA (1357 bp) into the polylinker (EcoRI-site) of the pTarget vector (5.67 kb) (Promega) [65] (figure 3A).

The human CST6-plasmid (hCST6) coding for cystatin M and the empty pTracer-CMV2 vector (6.2 kb; Invitrogen, Carlsbad, CA, USA) [66] were gifts from postdoc. Jon Briggs, Department of Tumour Biology, Rikshospitalet University Hospital HF (RUH-HF), Oslo, Norway. The plasmids were originally provided by Professor Daniel Keppler, Department of Cellular Biology & Anatomy, Louisiana State University Health Science (LSUHSC), USA. The CST6-plasmid contained the full length hCST6-cDNA (475 bp) subcloned into the KpnI/EcoRI sites in the polylinker of the mammalian expression vector pTracer-CMV2 (figure 3B).

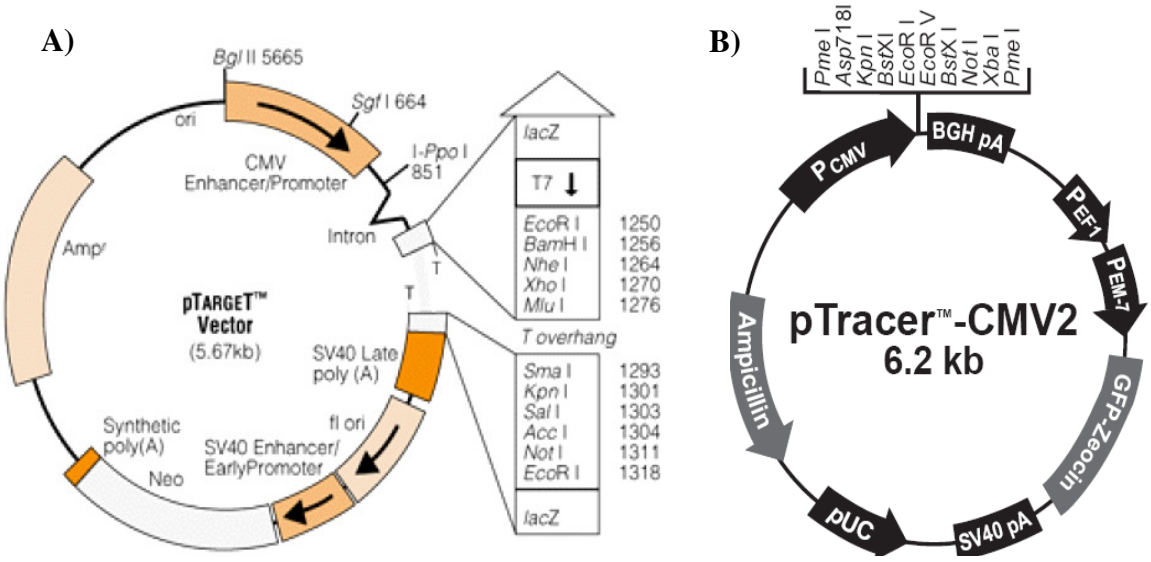

Figure 3: Cycle maps of pTarget (A) and pTracer-CMV2 (B) vectors. *The vector expressions of inserted genes is regulated by the human cytomegalovirus (CMV) promoter and terminated by simian virus (SV40) late poly adenylation signal (PA) and bovine growth hormone (BGH) PA, respectively. Both plasmids contain an ampicillin resistance gene.* ***A)*** *The pTarget vector has two plasmid subcloning sites: a T-vector (a linearized vector containing a thymidine at the 3`-ends) and a multiple cloning site (MCS). For generation of stable transfectants, the vector contains the neomycin phosphotransferase gene, expressed by SV40 enhancer promoter and terminated by a synthetic PA.* ***B)*** *The pTracer vector with cycle 3-GFP (green fluorescent protein) gene. For stable transfection, the vector contains a zeocin resistance gene, expressed by PEF1 and PEM-7 promoters and terminated by SV40 PA.*

In order to produce plasmids in high quantities, chemically competent *E. coli* were transformed (a 50 µl vial of One Shot® cells and 1-2 µg plasmid). For each plasmids, plasmid-transformed *E. coli* stocks in glycerol were frozen at -70 °C. In short, either directly after transformation or from glycerol stocks of *E. coli*, plasmids were isolated and purified (mini- and maxi-preps) in order to yield pure plasmids. The plasmids (2 µg) were digested (37 °C overnight) by suitable restriction endonucleases (EcoRI for LGMN and KpnI/EcoRI

for pTracer and CST6, respectively) and loaded on a 1 % agarose gel (at 100 V for 1-2 hours) parallel to a DNA-standard to verify the size of the inserted cDNAs and vectors (appendix).

4.2.1 Determination of plasmid concentration

The concentrations of the isolated plasmids were estimated by measuring optical density (OD). The absorbance was measured in a Gene Quant instrument. The samples were prepared by adding 180 µl distillated water to 2 µl of plasmid solution in a cuvette. The absorbance was measured at two wave lengths (260 and 280 nm) and the concentrations of DNA solutions were calculated using the following equation:

OD (at 260 nm) x 50 (DNA factor) x 90 (sample dilution factor) x 1000 = Plasmid concentration µg/µl

4.3 Transient transfection of HEK 293 cells

HEK 293 cells were used for transient transfection with LGMN- and CST6-plasmids. The transfection method used was based on liposome-mediated transfection by using lipofectamine 2000 (Invitrogen) as the lipid reagent. Liposomes are synthetic analogues of natural cellular membranes made of phospholipid layers. Phospholipids contain one water soluble end, and opposite water insoluble end, which allows complex formation with DNA and form spherical liposomes under aqueous conditions.

4.3.1 Transient transfection with LGMN-plasmid

Prior to transfection, HEK 293 cells were split, counted and seeded (0.3-0.5 x10^5 cells/well) onto six-well plates and incubated for 24-72 hours at 37 ^{o} C. A number of experimental adjustments were carried out to increase the efficacy of the transfection process (table 2). The main adjustments were titration of cell density, culturing in DMEM with or without serum and determining the proper amount of plasmid-DNA, as well as plasmid to liposome ratio. At the day of transfection, both plasmid and lipofectamine 2000 were diluted with DMEM, mixed gently and incubated at room temperature for 30 minutes. One ml of the lipid-plasmid complex was added to each well. The growth media was removed from the cells immediately before overlaying the lipid-plasmid complex (appendix).

Table 2: Experimental conditions used for transfection of LGMN-plasmid in HEK 293 cells.

Cell density ($x10^6$ cells)	Seeding time before transfection (days)	LGMN-plasmid concentration (µg)	Amount of Lipofectamine 2000 (µl)	µg DNA: µl Lipofectamin ratio	FBS in media	Antibiotics in medium	Transfection (TF) time (h)	Harvesting time after start of TF (h)
0.5, 1 and 2	1	5	20	1:4	Yes/No	Yes	48	48
0.5	1	1, 2.5, 5	20	1:20, 1:8, 1:4	Yes	Yes	48	48
0.5	1	1, 2.5, 5	10	1:10, 1:4, 1:2	Yes	No	24	48
0.3	3	2, 5	5	1:2	Yes	No	24	48, 72, 144
0.3	3	2.5, 5, 10, 20	5	1:2, 1:1, 2:1, 4:1	Yes/No	No	3, 6, 9, 24	48

4.3.2 Determination of transfection efficacy using maxFP-Green plasmid

In order to evaluate the efficacy of the transfection method (liposome-principle), the cells were transfected by maxFP-Green plasmid (figure 4), cloned from the copepod *Pontellina plumata* (a tiny crustacean that lives among plankton and is an important food source for many fish) [67]. Cells (5 $x10^5$ cells/well) were seeded onto six-well plates two days prior to transfection. Ten µl lipofectamine 2000 and 2.5 µg plasmid were diluted in DMEM without serum and antibiotics separately, mixed and incubated at room temperature for 30 minutes, before overlaying the cells. In the case of a successful transfection, the expressed fluorescent maxFP-Green will be visualized under fluorescence microscopy as dark green spots.

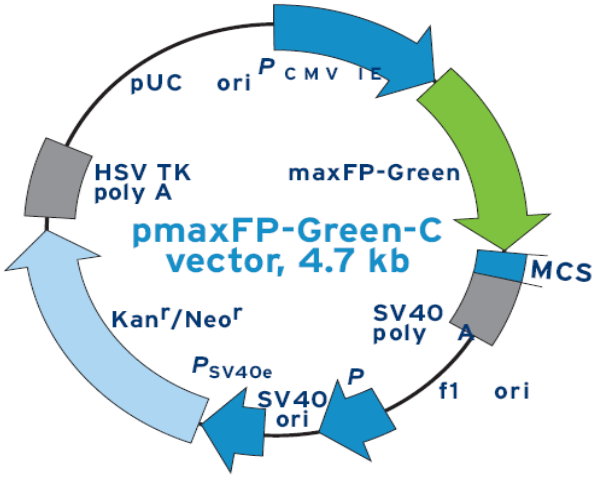

Figure 4: Cyclic map of maxFP-Green-C vector. *The vector uses the immediate early promoter of cytomegalovirus (CMV) to express the maxFP-Green plasmid (encoding the green fluorescent protein maxFP-Green). Plasmid expression is terminated by simian virus (SV40) (PA). The plasmid also contains kanamycin- and neomycin-resistance genes, expressed by bacterial promoter P (in bacteria) and SV40 early promoter in mammalian cells (for stable transfectants), respectively. The expression of antibiotic genes is terminated by Herpes simplex virus (HSV) thymidine kinase (TK) PA.*

4.3.3 Transient transfection with CST6-plasmid

HEK 293 cells (2.5-3 x10^5) were seeded onto six-well plates three days prior to transfection. The plasmids CST6 and pTracer (empty vector) (4 µg/well) as well as lipofectamine 2000 (10 µl/well) were diluted in DMEM without antibiotics and serum. The solutions were mixed gently and incubated at room temperature for 30 minutes before adding to the cells. The transfection solution was aspirated after 24 hours and HEK 293 medium was added. After 24 hours (48 hours after start of transfection), the media from the cells were collected and the cells were harvested and lysed in lysis buffer (appendix).

4.4 Protease activity measurements

The microplate reader Wallac Victor 3, 1420 Multilabel Counter (PerkinElmer), supplied with the Workout software was used under measurements of protease activity, total inhibitory activity against cysteine proteases and total protein concentration.

4.4.1 Proteolytic activity of legumain

The activity of legumain in whole cell lysates and elution fractions after size exclusion chromatography (4.8) was measured fluorimetrically using Z-Ala-Ala-Asn-NHMec as the specific substrate [9, 68]. Upon cleavage by legumain, the fluorescent leaving group – NH_2Mec caused increase in fluorescence.

Twenty µl of cell lysates or blank were added to a black 96-well microplate (Costar). After addition of 100 µl assay buffer with DTT (pH 5.8) (appendix) and 50 µl substrate (final concentration of substrate in the wells were 10 µM) by auto-injectors, a kinetic measurement based on increase in fluorescence (ΔF/s) over 120 minutes (some times 10 or 30 minutes) was carried out. The temperature was kept at 30 ^{o}C and all measurements were performed in triplicate. The excitation and emission wavelength were 360 and 460 nm, respectively (appendix).

4.4.2 Proteolytic activity of cathepsin B

The activity of cathepsin B was measured in the elution fractions from size exclusion chromatography (4.8). The activity was measured in the same way as legumain, but using the substrate Z-Arg-Arg-NHMec [44]. Twenty µl of the fractions were added to black 96-well plates and the temperature was kept at 30 ^{o}C in the Wallac Victor. After addition of 100 µl buffer (pH 5.5; appendix) and 50 µl substrate solution (final concentration of substrate in the wells were 20 µM) by auto-injectors, a kinetic measurement was performed and the increase in fluorescence per second (ΔF/s) was calculated over 10 minutes (appendix).

4.5 Measuring total inhibitory activity against cysteine proteases

To measure inhibitory activity against cysteine proteases, an indirect method was used which was established in an earlier work in our research group [69]. Inhibitory activity against cysteine proteases was measured by comparing the samples at the same degree of inhibition (50 %). A range of dilutions of media and a standard curve were used in order to estimate which dilution corresponded to a 50 % inhibition of papain (in media) and legumain (in fractions from 4.8). In this study, an inhibitory unit (IU) was defined as the concentration of inhibitory activity needed to inhibit 50 % of enzyme activity. Total inhibitory activity was calculated as IU/mg total protein for fractions, and IU/ml for media samples.

4.5.1 Total inhibitory activity against papain

The total inhibitory activity in media from CST6-transfected cells were determined by using papain as a target enzyme and measuring papain activity with the fluorescent synthetic peptide substrate Z-phenyl-arginin-NHMec. Before starting the assay, media samples were boiled (100 ^{o}C, 5 minutes) to inactivate any proteolytic enzymes present capable of cleaving the papain substrate (cystatins are resistant to high temperatures). Papain was diluted in 50 mM sodium acetate buffer (pH 4.5) and a 100 000 X dilution (appendix) was used in the

measurements, based on the previous work [69]. The measurements were performed using a weakly acidic phosphate buffer (papain assay buffer, pH 6.5) containing DTT. Black 96-well micro plate was used in this purpose, and the temperature was kept at 37 ^{o}C. Twenty µl of papain solution (100 000X) and 17 µl samples were added to the wells, except blank (20 µl sodium acetate + 17 µl papain assay buffer without DTT). Using auto-injectors, the wells were added 83 µl assay buffer and subsequently 50 µl substrate (final concentration 10 µM/well). Fluorescence (ΔF/s over 10 minutes) was measured at excitation and emission wavelength 355 and 460 nm, respectively (appendix).

4.5.2 Inhibitory activity against legumain

Using the same method as described for papain, inhibitory activity against legumain in media from CST6-transfected cells was measured. The fractions with highest legumain activity from analytical size exclusion chromatography (4.8) were used for this purpose. The fractions (20 µl) were added to black 96-well plates and 17 µl of medium from pTracer or CST6-transfected HEK 293 cells were added to the wells. Using auto-injectors, legumain assay buffer (appendix) was added (83 µl) to the wells followed by 10 minutes incubation time, before addition of 50 µl legumain substrate solution. The same protocol as for papain was used to perform a kinetic measurement based on ΔF/s over 10 or 120 minutes, when the temperature was kept at 30 ^{o}C.

4.6 Total protein determination

To measure total protein concentration in cell lysates, the Coomassie based procedure as described by Bradford was used [70]. The colour reagent (protein assay dye reagent concentrate) was diluted 1:5 in dH_2O and sterile filtered (appendix). The measurements were performed according to the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA) in the Wallac Victor, measuring absorbance at 595 nm. A standard curve was established by using bovine serum albumin (BSA, 50-300 µg/ml) for the calculation of concentration of proteins in cell lysates (appendix).

4.7 Immunoblotting

In order to prepare samples for electrophoresis analysis on SDS-polyacrylamide gels, proteins in cell lysates were concentrated by TCA precipitation (appendix). The total protein concentration was adjusted depending on results from the total protein measurements (4.6). Eventually, samples containing 10-20 µg total protein were run parallel to 2.5 µl of the

standard precision plus (Bio-Rad) on a 12 % SDS-polyacrylamide gel prior to blotting to a nitrocellulose membrane. After blotting for 45-60 minutes, the membrane was coloured by Ponceau S-solution, in order to confirm a successful blotting process. After washing by distillated water, the membrane was placed in T-TBS for 10 minutes. The membrane was blocked with 5 % non-fat dried milk in T-TBS prior to incubating with the primary antibody polyclonal rabbit anti human legumain (Abcam). The primary antibody was diluted in blocking solution (1:5000) as recommended by the manufacturer and incubated with the membrane overnight at 4 ^{o}C under gentle agitation. After washing in blocking solution, the membrane was incubated with the secondary antibody goat anti rabbit IgG HRP-conjugate (Bio-Rad) diluted in blocking solution (1:3000) for 1 hour. Then, the membrane was washed once by blocking solution and several times by T-TBS, before detection of immunoreactive bands by enhanced chemiluminescence ECL Western Blotting Detection System (Amersham Biosciences; appendix).

4.7.1 Quantification of Western bands

In order to quantify the bands obtained after the Western blotting analysis, images of the bands on a film was captured by GeneSnap scanner, version 6.05.01 (SynGene). The images were then densitometrically analyzed by using the automatic image analysis software GeneTools (SynGene). The software makes it possible to compare the graphical presentations of the different bands on the film by plotting the graphs in the same page. The relative differences in intensity of the bands are then calculated by determining the ratio of area between the bands of interest.

4.8 Analytical size exclusion chromatography

To investigate the molecular weight of proteins giving rise to the legumain (and cathepsin B) activity that appears in HEK 293, THP-1 and PC12 cells, the cell lysates were subjected to size exclusion chromatography. Samples (200 µl) were applied to a Superdex 75 10/30 column (Amersham Biosciences, Buckinghamshire, UK) equilibrated with buffer (appendix). Elution of the column was controlled by a FPLC-system, keeping flow to 0.5 ml/min and collecting fractions of 300 µl. Estimation of the molecular weight of proteins/activity eluting from the column was done by comparing with the following gel filtration standards (all from Amersham Biosciences): ribonuceases A (13.5 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and bovine serum albumin (67 kDa).

5. Results

5.1 Amplification, purification and verification of LGMN and CST6

In order to have enough plasmids to perform transfection experiments, the plasmids for LGMN, CST6 and the empty vector (pTracer-CMV2) were amplified and purified by using a JETSTAR plasmid purification kit (Genomed, appendix). To verify that the plasmids were intact, they were digested by restriction endonucleases (EcoRI for LGMN and KpnI/EcoRI for pTracer and CST6, respectively) overnight, before running on agarose gels (figure 5).

In the case of the LGMN-plasmid, gel electrophoresis revealed two distinct bands in the lane with the digested plasmid (figure 5A, lane 3), whereas only one band could be observed in the lane with undigested plasmid (lane 2). Empty pTarget vector was not available as control. By comparing the obtained bands from the digested plasmid (lane 3) with a DNA standard (lane 1), it was concluded that the lower band corresponded to a size of about 1300 bp and the upper band of approximately 5700 bp. The inserted LGMN-cDNA sequence consists of 1357 bp, and the pTarget vector of 5657 bp which is in agreement with the observed restriction bands.

For the CST6-plasmid, it was observed two bands in the lane with digested CST6 (figure 5B, lane 5) and only one band in the lanes with undigested pTracer vector, digested pTracer and un-digested CST6 (lanes 2, 3 and 4, respectively). By comparing the bands from the digested CST6-plasmid with the DNA-standard (lane 1), it was concluded that the lower band corresponded to the CST6-cDNA with a molecular size of 475 bp and the upper band to the linearized pTracer vector of 6.2 kb.

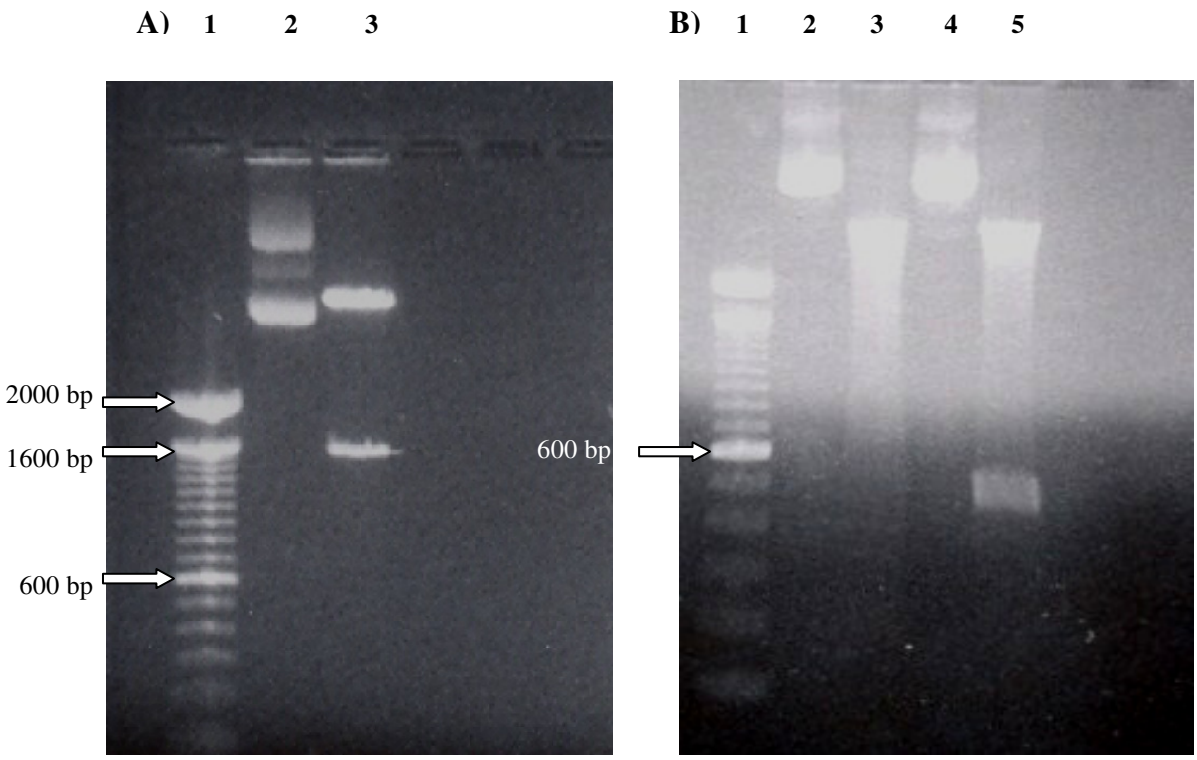

Figure 5: Agarose gel electrophoresis of purified LGMN (A) and CST6 (B) plasmids. *E. coli were transformed with plasmids, and the plasmids were isolated and purified using a JETSTAR plasmid purification system. The DNA (2 µg) were digested with restriction nucleases and run on 1 % agarose gel for 2 hours at 100 V.* ***A****) LGMN-plasmid. 1) DNA standard (100 bp DNA ladder); 2) Un-digested plasmid; 3) EcoRI-digested plasmid.* ***B****) CST6-plasmid. 1) DNA standard (100 bp DNA ladder); 2) Un-digested empty pTracer vector; 3) EcoRI/KpnI-digested pTracer; 4) Un-digested CST6-plasmid; 5) EcoRI/KpnI-digested-CST6. Arrows indicate sizes of bands in the DNA ladder.*

5.2 Transfection of HEK 293 cells with LGMN-plasmid

HEK 293 cells were transfected with the LGMN-plasmid to establish a transient and/or stable cell line which expresses high levels of legumain. As table 2 indicates, a number of experimental adjustments were performed in order to optimize the transfection conditions of HEK 293 cells with the LGMN-plasmid. The use of 20 µl liposomes was found to be toxic to the cells, especially when the liposome-plasmid complex was allowed to be in contact with the cells for 48 hours. The cells detached easily and formed loosely floating cell aggregates. The toxicity of liposomes was reduced when the transfection complex was not allowed to be in contact with the cells for more than 24 hours. However, the use of 5 µl liposomes, and in most cases 10 µl, tended to be preferred by the cells, despite long stimulating time (48 hours) with the transfection complex (not shown). The absence of serum from the growth media during culturing was also observed not to be preferred by the cells, as they detached easily from the flasks in the majority of experiments.

To evaluate transfection efficiency, legumain activity was measured in cell lysates. No increase in legumain proteolytic activity was observed in LGMN-transfected cells compared to controls (figure 6). Different amounts of LGMN-plasmid and liposomes did not cause any increase in legumain activity (figure 6A and B, respectively). Corresponding immunoblots of legumain verifying unsuccessful transfection are shown in figure 7, lanes 3 and 4. Only in one of many experiments using serum free media, a slight increase in legumain activity could be observed (figure 6C).

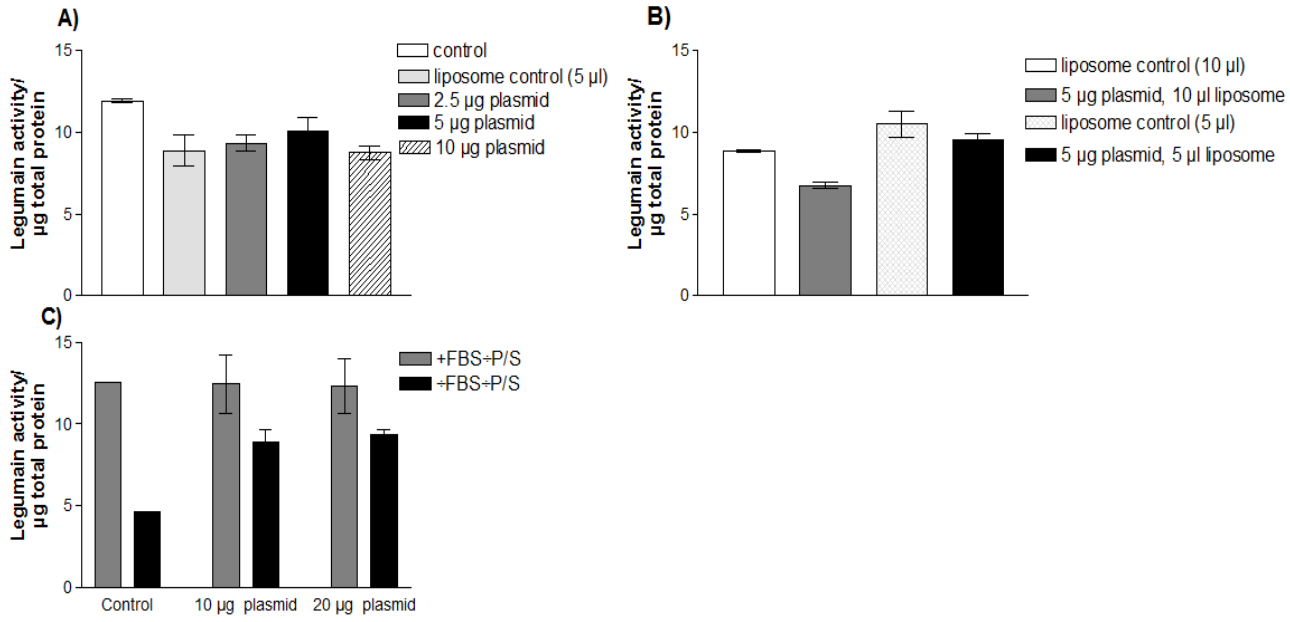

Figure 6: Proteolytic activity of legumain in HEK 293 cells transfected with LGMN-cDNA. A) *Cells (5 x 10^5) were seeded onto 6-well plates the day prior to transfection using 5 µl lipofectamine 2000 for 24 hours. The transfection medium (+FBS) was free from antibiotics. One representative experiment is shown (n=3).* ***B)*** *Cells (3 x 10^5) were seeded onto 6-well plates 3 days prior to transfection with 5 µg LGMN-plasmid. Transfection was performed using 5 or 10 µl lipofectamin 2000 (for 24 hours) and free from serum and antibiotics (n=2).* ***C)*** *Cells (5 x 10^5) were seeded onto 6-well plates the day prior to transfection. Transfection was aided by 5 µl lipofectamine 2000 (including liposome controls). The transfection media was either free from antibiotics (+FBS÷P/S), or from both serum and antibiotics (÷FBS÷P/S) (n=1). All cells were harvested and lysed 48 hours after start of transfection. The fluorogenic peptide Z-Ala-Ala-Asn-NHMec was used as the substrate for legumain and activity data were adjusted to µg total protein (see methods).*

5.2.1 Immunoblotting of legumain

To investigate whether transfection of HEK 293 cells with the LGMN-plasmid had resulted in a regulation of legumain protein expression, immunodetection of legumain in the cell lysates using an anti-legumain antibody (1:5000) was performed. The antibody gave three strong bands of approximately 57, 45 and 29 kDa, respectively (figure 7A). Molecular

weight of these bands was calculated using a protein standard (the standard precision plus, Bio-Rad). The 45 kDa band was composed of a double band. Untreated HEK 293 cells (control) showed high expression of all three legumain bands (lane 1). Consistent with the results from legumain activity measurements, it was no increase in intensity of the bands in the cell lysates from LGMN-transfected cells compared to controls. In one experiment, however, slight increase in band intensities could be seen in cell lysates of transfected cells with 5 or 10 µg LGMN-plasmid, respectively (figure 7A, lower bands in lanes 7 and 8). These bands (figure 7A, lower bands in lanes 5-8) were scanned and their intensities were determined by the automatic image analysis software GeneTools (SynGene; figure 3B). Increase in band intensities were 31, 80 and 131 % in cell lysates from HEK 293 cells transfected with 2.5, 5 and 10 µg LGMN-cDNA, respectively, compared to control.

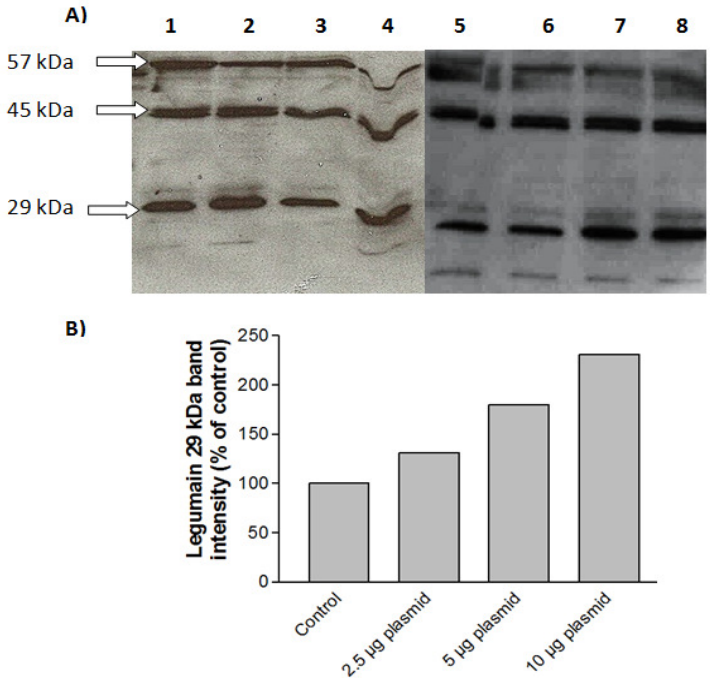

Figure 7: Representative immunoblots of legumain (A) and corresponding band intensity (B) in HEK 293 cell lysates transiently transfected with LGMN-cDNA. A) *Transfection was aided by lipofectamin 2000. The cell lysates were TCA precipitated and 12.5 µg total protein were loaded in each well. (1) Control (untreated). (2) Cells transfected with 1 µg plasmid. (3) Cells transfected with 2.5 µg plasmid. (4) Cells transfected with 5 µg plasmid. Lanes 2-4; 10 µl liposome. (5) Liposome control. (6) Cells transfected with 2.5 µg plasmid. (7) Cells transfected with 5 µg plasmid. (8) Cells transfected with 10 µg plasmid. Lanes 5-8; 5 µl liposome.* ***B)*** *Intensities of the lower bands (29 kDa) in A (lanes 5-8), determined by automatic image analysis software; GeneTools (SynGene). The intensities are given as percent of control (liposome treated).*

5.3 HEK 293 cells were efficiently transfected by liposomes

Since the method was not previously established in the laboratory and the legumain activity data and Western blots in HEK 293 cells transfected with the LGMN-plasmid showed no regulation of legumain, it was desirable to check the transfection efficacy by using maxFP-Green-plasmid (Amaxa) and lipofectamin 2000. Importantly, it was necessary to test the technical aspects under performance of transfections.

HEK 293 cells were transfected by 2.5 µg maxFP-Green and 10 µl liposomes without serum and antibiotics, and the transfection complex was changed with fresh medium 24 hours after start of transfection. Upon expression of the fluorescent maxFP-Green protein, the cells can be visualized as green cells under fluorescence microscopy. The transfection efficacy of HEK 293 cells with maxFP-Green plasmid appeared to be 70-80 % (figure 8) determined by green versus colourless cells. That indicated that the transfection method and optimized conditions used during the experiments were sufficient for successful transfection.

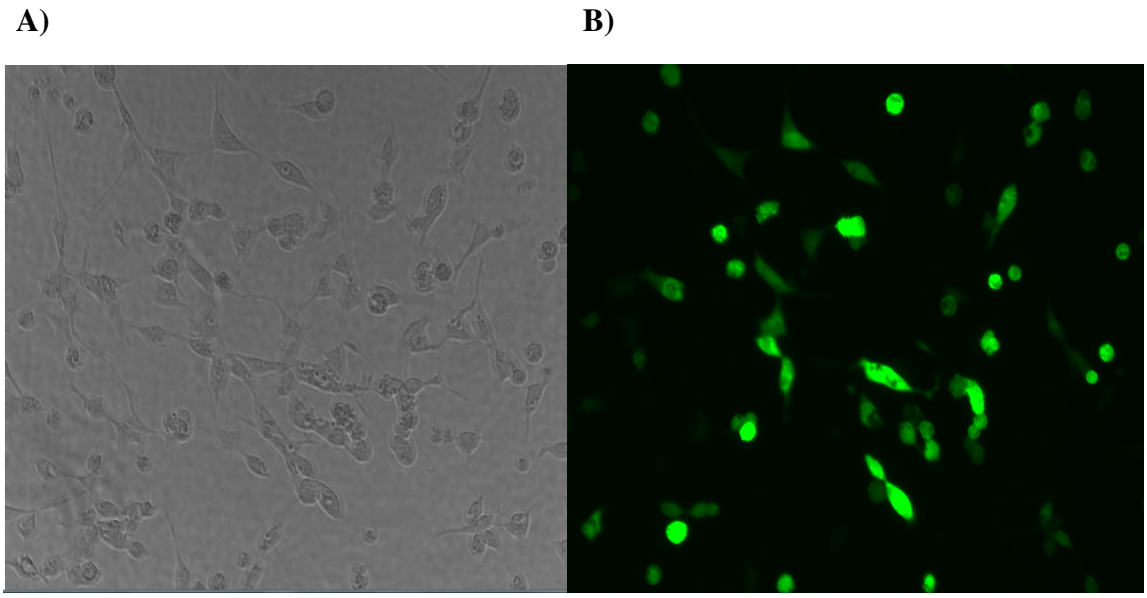

Figure 8: Evaluation of the transfection efficacy of HEK 293 cells by using maxFP-Green plasmid. *Cells (5 x10^5) were seeded onto 6-well plates 2 days prior to transfection with 2.5 µg maxFP-Green plasmid using 10 µl lipofectamine 2000 and medium without serum and antibiotics. The transfection complex was changed with fresh medium 24 hours after start of transfection and the cells were visualized 48 h after start of transfection.* ***A)*** *A section of cells visualized by light microscopy (enlarged 10 x).* ***B)*** *The same section of the cells visualized by fluorescence microscopy (10 x). The green spots seen in (B) are the expressed maxFP-Green protein (cloned from Pontellina plumata).*

5.4 Over-expression of cystatin M

5.4.1 Over-expression of cystatin M in CST6-transfected HEK 293

In spite of negative results from the transfection experiments of LGMN-plasmid in HEK 293 cells, the cells were transfected with CST6-plasmid. The purpose of this opposed strategy was to generate a cystatin M over-expressing cell line, and to characterize the influence of cystatin M on legumain activity.

Total inhibitory activity against papain in the conditioned media from CST6- and pTracer-transfected HEK 293 cells was measured in order to investigate if the transfected cells had expressed and secreted functional cystatin M. In all experiments performed (n=3), the total inhibitory activity (IU/ml) against papain in media from CST6-transfected cells was higher than in media from pTracer-transfected cells (figure 9). The increase in total inhibitory activities varied and was 36, 75 and 104 %, respectively.

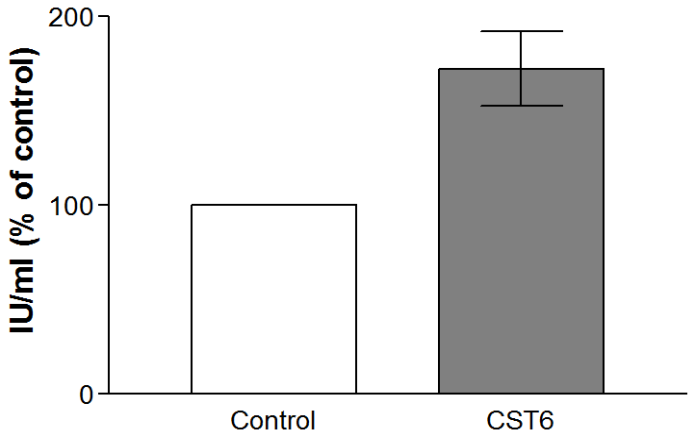

Figure 9: Total inhibitory activity against papain in media from CST6- and pTracer-transfected HEK 293 cells. *Cells (2.5-3 x10^5) were seeded 3 days prior to transfection with 4 µg pTracer (control; open bars) or 4 µg CST6 (closed bars) plasmids using 10 µl lipofectamine 2000 for 24 hours. The transfection media was free from both FBS and P/S. Media and cells were harvested 48 h after start of transfection. Total inhibitory activity (IU/ml) was measured fluorimetrically using papain as the target enzyme and is given as percent of control (empty vector; pTracer) (n=3).*

5.4.2 Over-expression of cystatin M in CST6-transfected melanoma cells

A panel of melanoma cell lines (table 1) were transfected with CST6 or pTracer by postdoc. Jon Briggs at RUH-HF. The aim was to study over-expression of cystatin M in these cells, and its correlation with legumain activity. Media from the established melanoma cell lines A-375, FEMX-I, HHMS and SKMEL-28, and the malignant melanoma cell lines isolated from patients MM 11, MM 35 and MM 69b, all transfected with CST6 or empty vector

(pTracer), were received for measurement of total inhibitory activity against cysteine proteases. Over-expression of cystatin M was observed in media from all melanoma cell lines transfected with CST6-plasmid when compared to pTracer-transfected cells (figure 10). Among these cell lines, CST6-transfected SKMEL-28 showed highest increase in total inhibitory activity (IU/ml) of 41 % followed by MM 11 with 39 % compared to control. In MM 69b cells, however, over-expression of cystatin M was minimal (figure 10).

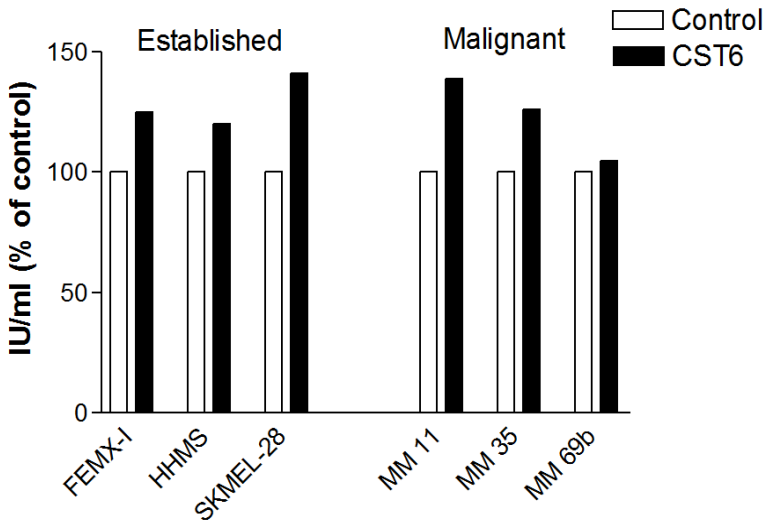

Figure 10: Total inhibitory activity against papain in media from established melanoma and malignant melanoma cell lines transfected with CST6 or pTracer-plasmid. *The established melanoma cells lines FEMX-I, HHMs and SKMEL-28, and the malignant melanoma cell lines MM 11, MM 35 and MM 69b were transfected using 4 µg pTracer (control; open bars) or CST6 (closed bars) and 10 µl lipofectamine 2000. Total inhibitory activity (IU/ml) against papain was measured in the conditioned media. Total inhibitory activity data are given as percent of control (pTracer). The transfections were performed by postdoc. Jon Briggs at RUH-HF.*

5.5 Regulation of legumain activity in CST6-transfected cells

Despite the fact that cystatin M is a secreted protein and legumain is an intracellular protein located in lysosomes, it was interesting to investigate if there was any correlation between legumain activity and cystatin M in CST6-transfected cells. Thus, legumain activity was investigated in cell lysates from HEK 293 and MM 35 cells transfected with CST6, respectively.

5.5.1 Large decrease of legumain activity in CST6-transfected HEK 293 cells

Measuring legumain activity in cell lysates from CST6-transfected HEK 293 cells showed remarkable decrease in activity (46-94 %) when compared to pTracer-transfected cells (n=5) (figure 11). The average decrease of legumain activity in the CST6-transfected cells was 79

%. Obvious association between high total inhibitory activity in media from the transfected cells (figure 9) and decrease in legumain activity in the cells was observed (table 3).

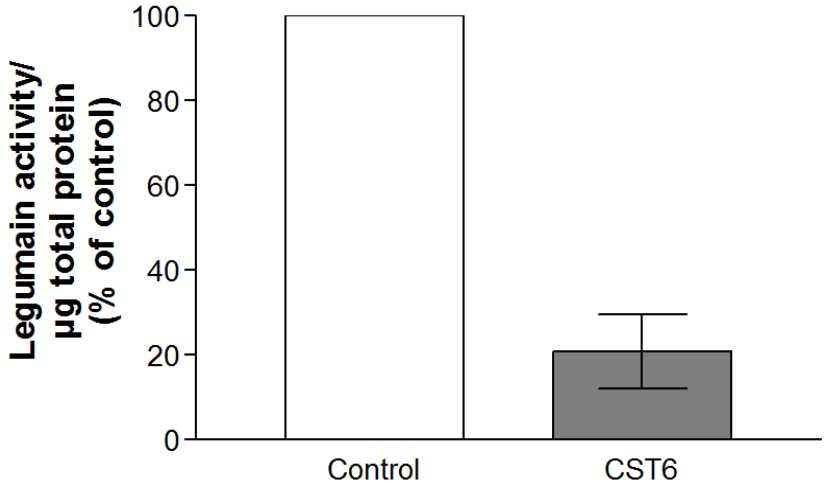

Figure 11: Legumain activity in cell lysates from pTracer- or CST6-transfected HEK 293 cells. *Cells (2.5-3 x10^5) were seeded 3 days prior to transfection with 4 µg empty vector (pTracer; control) (open bars) or 4 µg CST6 (closed bars) by using 10 µl lipofectamine 2000 for 24 hours. Media and cells were harvested 48 hours after start of transfection. Legumain activity was measured fluorimetrically by using the substrate Z-Ala-Ala-Asn-NHMec and activity data were adjusted to µg total protein. The activity data are given as percent of control (pTracer) (n=5).*

Table 3: Correlation between increase in total inhibitory activity in media and decrease in legumain activity of CST6-transfected HEK 293 cells. *Cells (2.5-3x10^5) were seeded 3 days prior to transfection with 4 µg empty vector (pTracer) or 4 µg CST6 using 10 µl lipofectamine 2000 for 24 hours. Media and cells were harvested 48 h after start of transfection. Five independent experiments are shown (n.a = not analyzed).*

CST6-transfection no.	% increase in total inhibitory activity (IU/ml) in cell media	% decrease in legumain activity in corresponding cell lysates
1	36	46
2	75	80
3	n.a	81
4	104	94
5	n.a	94

5.5.2 Moderate decrease of legumain activity in CST6-transfected melanoma cells

Legumain activity was also measured in cell lysates from the CST6-transfected established melanoma cell lines A-375 and FEMX-I, and the malignant melanoma cell line MM 11. There was observed decrease of legumain activity in all the CST6-transfected cell lines

(figure 12). The decrease in legumain activity was 41 % in A-375 compared to control (pTracer-transfected). In two independent experiments with FEMX-I (A and B), the decrease in legumain activity was 13 and 18 %, respectively. Decrease of legumain activity in MM 11 cells (A and B) was 10 and 20 %, respectively, in two independent experiments (figure 12). There was observed a slight correlation between increase in total inhibitory activity in media and decrease of legumain activity in CST6-transfected melanoma cells (not shown).

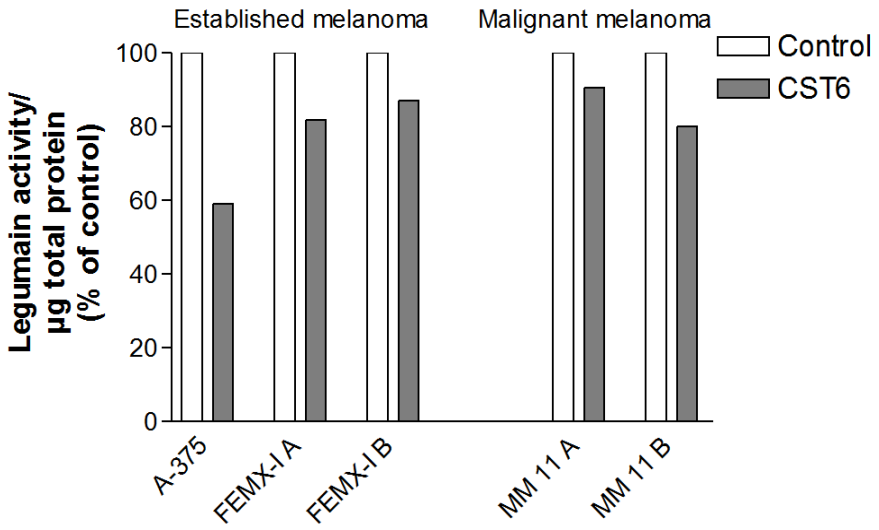

Figure 12: Legumain activity in CST6-transfected melanoma cells. *The established melanoma cell lines A-375 (n=1) and FEMX-I (n=2; A and B), and the malignant melanoma cell line MM 11 (n=2; A and B) were transfected with 4 µg empty vector (pTracer; control; open bars) or CST6 (closed bars) using 10 µl lipofectamin 2000. The transfections were performed by postdoc. Jon Briggs at RUH-HF and cell lysates received for enzymatic analysis. Legumain activity was measured fluorimetrically and the activity data were adjusted to µg total protein. Activities are given as percent of control (pTracer).*

5.6 Regulation of legumain activity after exposure of living cells to cystatin M-conditioned media

Although cystatin M and legumain locate differently in the cells, it was desirable to investigate whether addition of cystatin M exogenously had any impact on intracellular legumain activity. For this purpose, HEK 293, THP-1 and PC12 cells were cultured and treated by cystatin M-conditioned media from CST6-transfected HEK 293, SKMEL-28 and MM 35 cells, respectively.

5.6.1 Legumain activity decreased in HEK 293 cells

HEK 293 cells were treated by undiluted cystatin M-conditioned medium from CST6-transfected HEK 293 cells and legumain activity in the cell lysates was measured. Legumain

activity varied and decreased (12-50 %) in all experiments performed (figure 13) with an average decrease of legumain activity of 26 %. There was a correlation between high total inhibitory activity in the conditioned medium used and decrease in legumain activity (table 4).

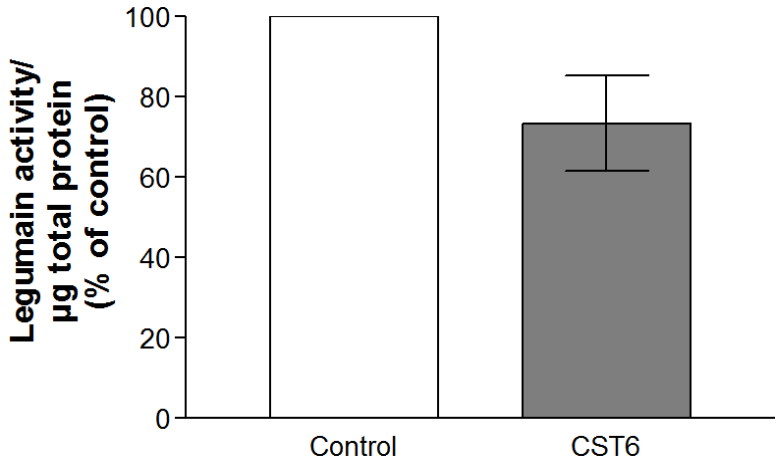

Figure 13: Legumain activity in HEK 293 cells treated with cystatin M-conditioned media from CST6-transfected HEK 293 cells. *Cells ($3x10^5$) were seeded onto 6-well plates 3 days prior to treatment with 800 µl control cell media (pTracer; open bars) or cystatin M-conditioned media (closed bars). The cells were harvested and lysed 24 hours after treatment with conditioned media. The activity data are given as percent of control (pTracer) and adjusted to µg total protein (n=3).*

Table 4: Correlation between increase in total inhibitory activity in conditioned media and decrease in HEK 293 cells intracellular activity of legumain. *Cells ($3x10^5$) were seeded onto 6-well plates 3 days prior to treatment with 800 µl control cell media (pTracer) or cystatin M-conditioned media. The cells were harvested and lysed 24 hours after treatment with conditioned media. Legumain activity, and total inhibitory activity were measured in cell lysates and conditioned media, respectively. The data are given as percent of control (empty vector; pTracer). Three independent experiments are shown.*

Experiment no.	% increase in total inhibitory activity (IU/ml) in cystatin M-conditioned media	% decrease in legumain activity in cell lysates
1	36	12
2	75	18
3	104	50

5.6.2 No regulation of legumain activity in THP-1 cells

Large increase (approximately 50-fold) in legumain activity was observed when THP-1 cells were allowed to grow for ten days after initially 24 hours stimulation with PMA (figure 14). PMA-stimulated THP-1 cells were treated with cystatin M-conditioned medium (diluted 1:3 in THP-1 medium) for 3, 6 and 10 days, respectively. The conditioned media used for this

purpose were collected after transfection of HEK 293 cells by CST6- and pTracer-plasmids. Only in one of three experiments performed (n=3), a 56 % decrease in legumain activity was observed when the cells were treated for 3 days with cystatin M-conditioned medium (with 36 % increase in total inhibitory activity) compared to control medium (figure 14A). No regulation of legumain activity was observed in the other experiments with 75 and 104 % increase in total inhibitory activity in conditioned media, respectively (figure 14B and C).

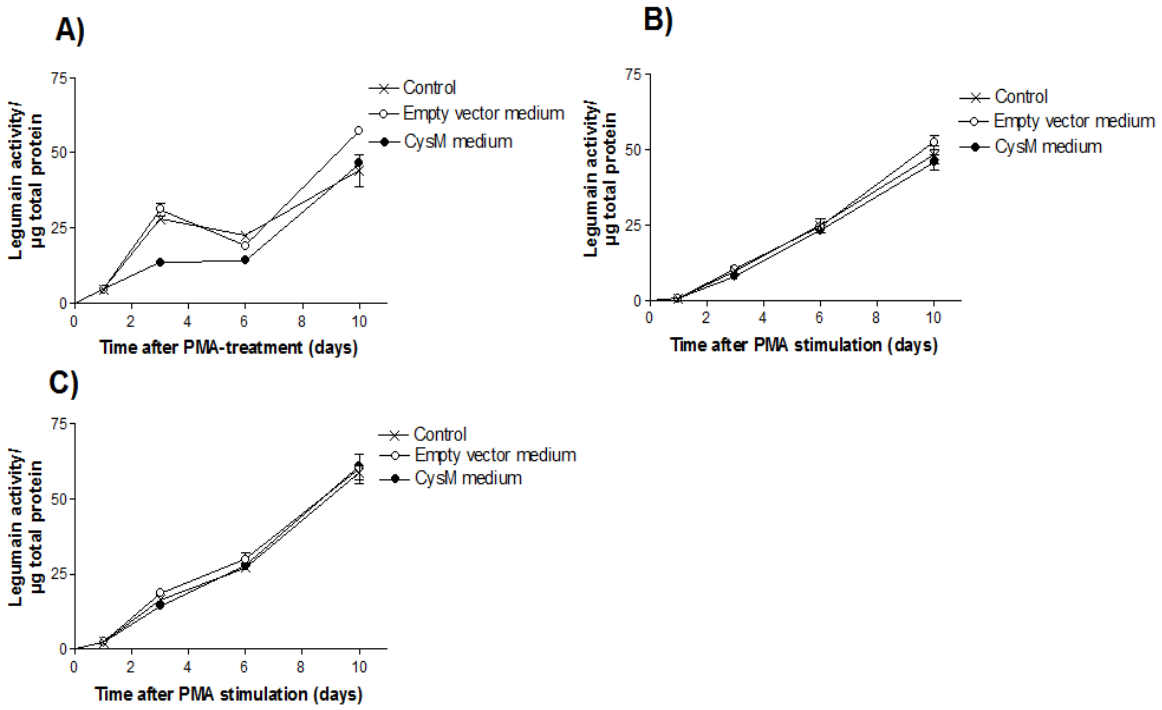

Figure 14: Time-response of legumain activity in PMA-stimulated THP-1 cells treated with cystatin M-conditioned media*. Cells (5x10^5) were seeded onto 6-well plates and treated with 40 ng/ml PMA for 24 hours. After washing (day 0), the cells were allowed to grow for 3, 6, and 10 days. Three days before harvesting, the cells were treated either by cystatin M-conditioned media from CST6-transfected or control cell media (pTracer), both diluted 1:3 with THP-1 medium (total 1 ml/well). Controls were treated by THP-1 media only. Data from three independent experiments are shown (n=3). Experiments* ***A), B)*** *and* ***C)*** *represent stimulation by cystatin M-conditioned media with 36, 75 and 104 % increase in total inhibitory activity, respectively. Legumain activity was measured in cell lysates and the activity data were adjusted to µg total protein.*

5.6.3 No regulation of legumain activity in PC12 cells

PC12 cells were treated by cystatin M-conditioned media from CST6-transfected SKMEL-28 or MM 35 cells (both diluted 1:1 in PC12 medium) and legumain activity was measured in cell lysates. No regulation in enzymatic activity of legumain could be observed upon addition of cystatin M-conditioned medium to these cells (figure 15). Increases in total inhibitory

activity in the cystatin M-conditioned media used were 39 and 26 % for SKMEL-1 and MM 35, respectively.

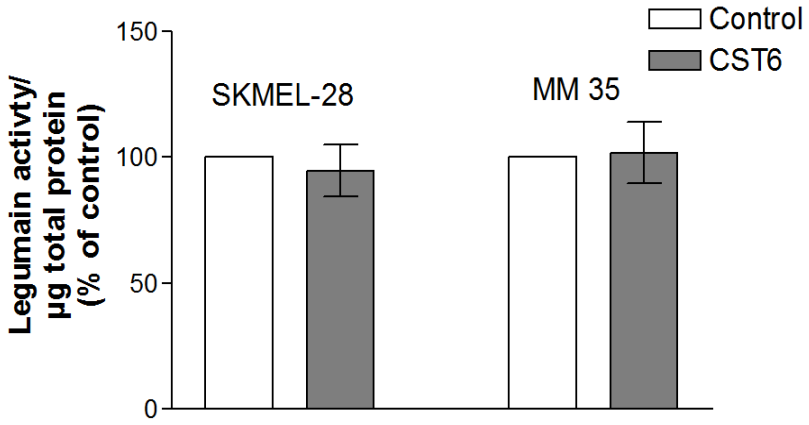

Figure 15: Legumain activity in PC12 cells treated by cystatin M-conditioned media*. Cells (1.5x10^5) were seeded onto 6-well plates 4 days prior to addition of cystatin M-conditioned medium diluted 1:1 in PC12 medium. Conditioned medium from CST6- (closed bars) or pTracer- (open bars; controls) transfected SKMEL-28 or MM 35 cells were used (increase in total inhibitory activity in medium from CST6-transfected cells was 39 and 26 %, respectively). After 24 hours stimulation, the cells were harvested and lysed. Legumain activity was measured in cell lysates and activity data were adjusted to µg total protein. The activity data are given as percent of control (empty vector).*

5.7 Analytical size exclusion chromatography of cell lysates from HEK 293, THP-1 and PC12 cells

5.7.1 Molecular weights of legumain and cathepsin B activities

Cell lysates from HEK 293 and PC12 cells, as well as PMA-stimulated THP-1 cells (40 ng/ml, 24 hours) grown for 10 days, were exposed to size exclusion chromatography in order to characterize the molecular weights of proteins responsible for the legumain and cathepsin B activities in the cells. To find the main legumain and cathepsin B activity fractions, the enzyme activities in different fractions were measured (figure 16). Legumain activity varied in the cell lines studied and the molecular weight of the fraction (fractions) having highest legumain activity was found to be different (figure 16A). In HEK 293 cells, legumain activity eluted in fraction 15 corresponding to a molecular weight of approximately 37 kDa. Contrary, in THP-1 cells, fraction 18 (molecular size of approximately 25 kDa) had the highest legumain activity. In addition, PC12 cell fraction 16 (approximately 32 kDa) possessed the highest legumain activity (figure 16A).

Cathepsin B activity in the same fractions was measured as well, in order to investigate the correlation with those having highest legumain activity. Very little or no cathepsin B activity was observed in HEK 293 cells compared to THP-1 and PC12 cells (figure 16B). Fraction 17 with a molecular weight of approximately 29 kDa in HEK 293 and THP-1 cells, and fraction 16 (approximately 32 kDa) in PC12 cells possessed highest cathepsin B activity (figure 16B).

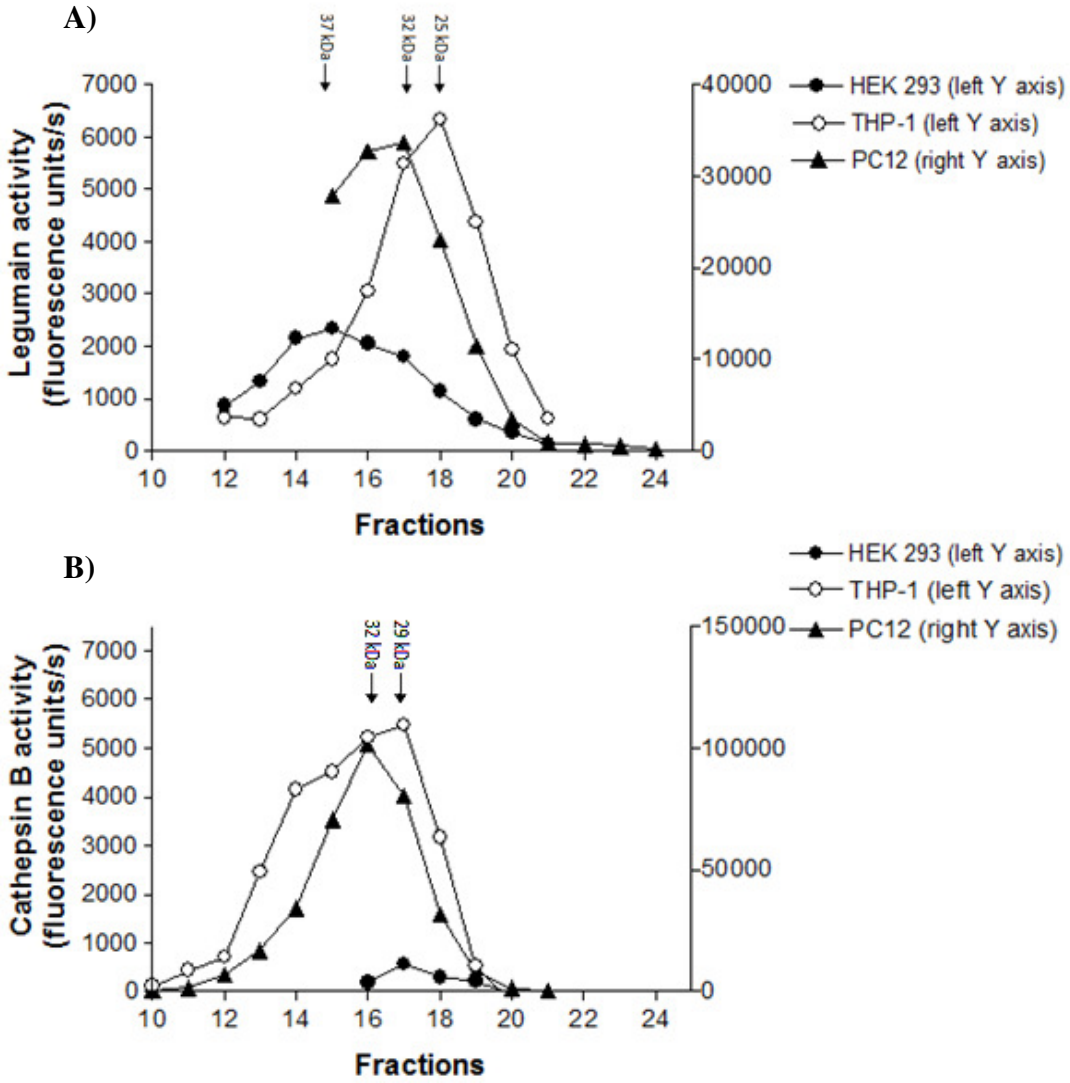

Figure 16: Molecular weight and enzymatic activities of legumain (A) and cathepsin B (B) in cell lysate fractions from size exclusion chromatography*. A bulk of HEK 293 and PC12 cells were cultured, harvested and lysed. THP-1 cells were stimulated by PMA (40 ng/ml, 24 hours), washed and cultured for 10 days. Cell lysates (200 µl) were passed through a Superdex 75 (10 – 30) column and fractions of 300 µl were collected.* ***A)*** *Legumain activity in HEK 293, THP-1 and PC12 cells. Fractions 12-21 for HEK 293 and THP-1 cells, and PC12 fractions 15-24 were measured. Results from one representative experiment are shown (n=2).* ***B)*** *Cathepsin B activity in HEK 293, THP-1 and PC12 cells. Fractions 10-22 are measured in all cell lines (n=1). Arrows indicate molecular weights of highest legumain (A) and cathepsin B (B) activity fractions.*

5.7.2 Inhibition of legumain activity fractions after size exclusion chromatography

Since legumain activity fractions in the cell lines studied (HEK 293, THP-1 and PC12 cells) had different molecular weights, it was interesting to investigate whether cystatin M or other known cysteine protease inhibitors affected legumain activity in these cell fractions. Cystatin M-conditioned media from HEK 293 cells transfected with CST6, as well as the cathepsin B-specific inhibitor CA074 and the cathepsins inhibitor E64 were used to study the inhibitory profile of the highest legumain activity fractions of HEK 293, THP-1 and PC12 cells, respectively.

Cystatin M-conditioned medium caused inhibition of legumain activity in all the cell lines studied (figure 17A). However, the inhibition varied between the different cell lines. Legumain activity fractions from HEK 293 and PC12 showed almost similar inhibitory characteristics and were greatly inhibited by cystatin M-conditioned medium. On the contrary, the legumain activity fraction of THP-1 cells treated with cystatin M-conditioned medium appeared to be less affected. The increase in total inhibitory activity in the cystatin M-conditioned medium used to treat all the cell lines was 104 % compared to control medium (pTracer-transfected). To inhibit 50 % of legumain activity in PC12, HEK 293 and THP-1 cells, 1, 2 and 23 % dilution of cystatin M-conditioned media, respectively, were needed.

In addition, media from HEK 293 cells transfected with empty vector (pTracer; control) or CST6 were compared (figure 17B). HEK 293 and PC12 cells were greatly affected by the contribution from cystatin M-conditioned medium when compared to control medium. In order to inhibit 50 % activity of the legumain fraction from HEK 293 cells, a 5 % dilution of cystatin M-conditioned compared to a 47 % dilution of control medium were needed (figure 17B). In PC12 cells, a 4 % dilution of cystatin M-conditioned compared to a 76 % dilution of control medium were needed to inhibit 50 % of legumain activity. Whereas in THP-1 cells, a 6 % dilution of both cystatin M-conditioned and control medium were needed, this means that the contribution from cystatin M did not affect legumain activity.

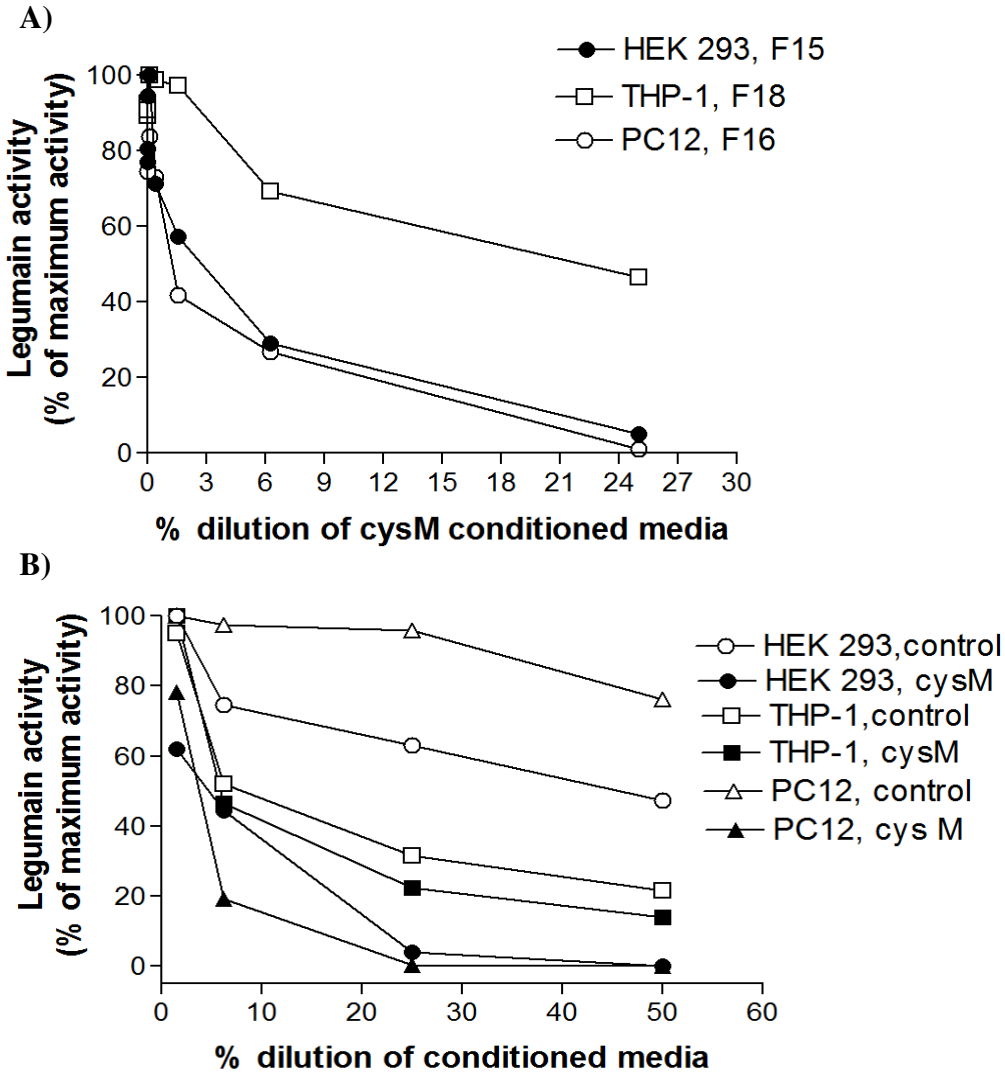

Figure 17: Inhibition of enzyme activity in legumain fractions of HEK 293, THP-1 and PC12 cells by cystatin M-conditioned media (A) and contribution of cystatin M to the inhibition (B). *HEK 293 and PC12 cells were cultured, harvested and lysed. THP-1 cells were stimulated by PMA (40 ng/ml, 24 hours) washed and cultured for 10 days. Cell lysates (200 µl) were passed through a Superdex 75 (10 – 30) column and fractions of 300 µl were collected. Increase in total inhibitory activity in the cystatin M-conditioned medium used was 104 %.* ***A)*** *Medium (containing serum) from CST6-transfected HEK 293 cells was diluted in serum free media and 17 µl was added to the respective legumain fractions (F; 20 µl) in black 96-well plates (n=1).* ***B)*** *Diluted cystatin M-conditioned and control (empty vector) media were used (n=1). Legumain activity was measured using the substrate Z-Ala-Ala-Asn-NHMec. Activity data are given as percent of maximum activity.*

Moreover, legumain activity in the same fractions as above was measured after addition of the cathepsin-inhibitors E64 and CA074. Legumain in HEK 293 and PC12 cells were unaffected by these inhibitors, while legumain in THP-1 cells showed a large decrease in activity of 85 and 79 %, respectively, when E64 or CA074 were added (figure 18). Contribution of cystatin M to the total inhibitory activity against legumain was calculated as

the difference between activities obtained after additions of control cell media (pTracer) or cystatin M-conditioned media, respectively, (figure 17B) at the same dilution (1:4 dilution) of media.

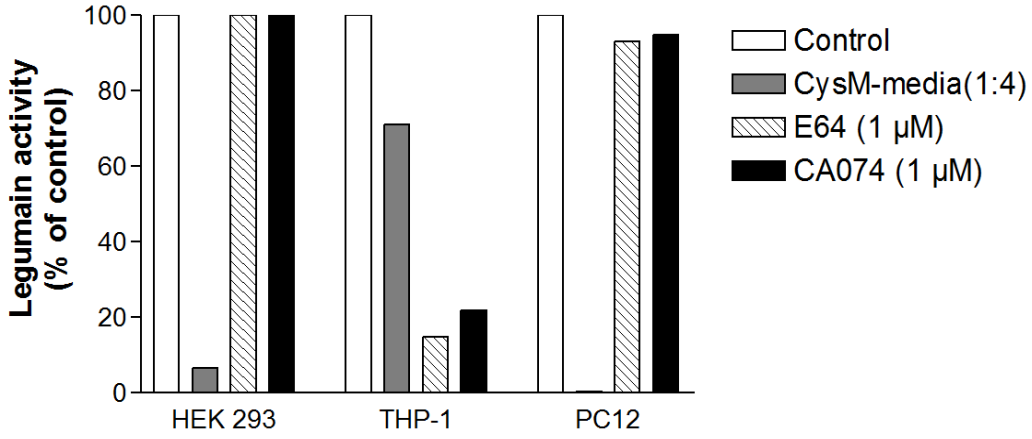

Figure 18: Effect of the cysteine protease inhibitors cystatin M, E64 and CA074 on legumain activity in HEK293, THP-1 and PC12 cell lysate fractions. *HEK 293 and PC12 cells were cultured, harvested and lysed. THP-1 cells were stimulated by PMA (40 ng/ml, 24 hours), washed and cultured for 10 days. Cell lysates (200 µl) were passed through a superdex 75 (10 – 30) column and fractions of 300 µl were collected. Cystatin M-conditioned media (increase in total inhibitory activity of 104 %) from CST6-transfected HEK 293 cells was diluted 1:4 in serum free media and added to the highest legumain activity fractions. E64 and CA074 (1 µM in dH_2O) were added to the wells containing 20 µl of fractions in black 96-well plates. Legumain activity was measured by using the substrate Z-Ala-Ala-Asn-NHMec. Activity data are given as percent of control (un-treated).*

6. Discussion

6.1 Establishment of a transfection method

The major objective of this study was to establish a method in our laboratory for transient transfection of rLGMN- or alternatively hCST6-plasmid, encoding rat legumain and human cystatin M, respectively. Initially, HEK 293 cells were transfected with rLGMN-plasmid using liposomes as the transfection reagent. HEK 293 cells were chosen as the host for transfection, since these cells are described as being easily transfectable using liposomes [71] and have previously been successfully transfected with human legumain [37, 72]. A number of adjustments were made to optimize transfection efficacy with the LGMN-plasmid. The most important of these were titration of number of cells, concentration of plasmid and amount of liposomes. As transfections were performed in 6-well plates, seeding $5x10^5$ cells/well one day before transfection or $2.5x10^5$ cells/well three days prior to transfection appeared to be most suitable to obtain optimal confluence (80-90 %). Using 20 µl liposomes appeared to be toxic to the cells, as cells detached from the culturing flasks. Decreasing liposome amount (10 and 5 µl) resulted in less toxicity, especially when 5 µl liposome was used. Among other adjustments, transfection was performed in serum and antibiotic containing media versus absence of both or one of these. There might be a possibility that antibiotics and contents in serum prevent the DNA plasmid from complex formation with liposomes and most protocols are free from both serum and antibiotics. Our cells detached easily when cultured without serum, therefore serum was initially preferred during transfections. Splitting and culturing in media with serum three days before transfection and subsequently transfection and growing in serum and antibiotic free media did not cause any detachment of the cells. In this way, the cells were more tightly attached to the culturing flasks before transfection process than seeding only one day prior to transfection followed by growing in serum free media. Transfection time (3, 6, 9, 24 and 48 hours) with the transfection complex (LGMN-plasmid and liposome) was also one of the parameters tested under the adjustments.

To evaluate whether the transfection had caused any increase in legumain expression and/or increase in enzymatic activity, immunoblotting and measurements of enzymatic activity were performed. Since empty pTarget vector was not available, untreated or liposome treated cells

were used as controls. Despite the adjustments made to achieve an optimal transfection efficacy, no indications of legumain over-expression could be observed in lysates from the LGMN-transfected HEK 293 cells. However, in two of many comparable experiments, when serum was not used during transfection and cells were seeded three days prior to transfection, an increase in legumain activity was observed (75 and 20 % compared to controls, respectively). These latter incidents, however, were rare observations as similar experiments did not confirm these.

The unsuccessful transfection of HEK 293 cells with the LGMN-plasmid, despite all attempts of adjustments, made it necessary to check transfection efficacy of these cells using maxFP-Green-plasmid and liposomes. Importantly, it was necessary to test the technical aspects under performance of transfections. Fluorescence microscopy verified that the cells were efficiently transfected. This confirmed that the transfection method and optimized conditions used during the experiments were sufficient for successful transfection.

Although unsuccessful transfection of the LGMN-plasmid, HEK 293 cells were transfected with the hCST6-plasmid (encoding human cystatin M/E, a potent inhibitor of legumain). To investigate whether the cells were successfully transfected, the increase in total inhibitory activity (IU/ml) against cysteine proteases in media was measured using papain as the target enzyme. In all the CST6-transfected cells total inhibitory activity increased but varied between experiments. This verified that the transfection method was effective for CST6.

Since the transfection complex (CST6-plasmid and lipofectamin) was removed and changed with fresh media (with or without serum) after 24 hours, the secreted cystatin M in the analyzed media was from the last 24 hours before harvesting only. To determine the contribution of cystatin M to the total inhibitory activity in media, control cell media (pTracer; empty vector) and cystatin M-conditioned media from CST6-transfected cells were compared. In the majority of experiments, both media samples contained 10 % serum which contains a wide range of other endogenous substances including cystatins. In two experiments, however, media samples did not contain serum and the observed total inhibitory activity in control cell media (empty vector; pTracer) was assumed to be the basal inhibitory activity of secreted cystatins C, M/E and F to the media. When media from CST6- and pTracer-transfected cells were compared, any increase of total inhibitory activity observed was due to the contribution from over-expressed cystatin M.

Since only the LGMN-plasmid failed to be transfected, possible reasons had to be discussed. One of the possible reasons to the failure of LGMN-transfection could be the large size of the LGMN-plasmid of approximately 7 kb. Since the CST6-plasmid (approximately 6.7 kb) and the maxGFP-Green-Plasmid (4.7 kb) were successfully transfected in HEK 293 cells, the hypotheses about impact of insert size became less probable. Based on previous findings, it was apparent that the cells were able to internalize the LGMN-plasmid-liposome complex and the transfection process was not the reason of failure of legumain over-expression. In a corresponding study, on the contrary, HEK 293 cells successfully over-expressed legumain when the cells were transfected with mouse legumain-cDNA [43]. In view of the fact that no gene sequencing data was provided when the LGMN-plasmid was received, it is likely that the LGMN-plasmid contained mutations which prevented successful transcription and translation of legumain in HEK 293 cells. This is possible since the construction of the LGMN-cDNA was performed by polymerase chain reaction (personal communication: Kazumi Ishidoh). Gene sequencing of the received LGMN-plasmid, however, could be performed but has not been included due to time limit of this study.

6.2 Molecular weight of active legumain

It has been shown that legumain in order to be activated must go through two successive cleavages of C- and N-terminal prepro-proteins, respectively [46]. Legumain is translated as a preproform, transferred through the Golgi apparatus as the proform (56 kDa), and localized in lysosomes as the mature active (46 kDa) enzyme [47]. This activation is autocatalytic requiring acidic pH [11, 46]. Active legumain in living cells has been shown to possess a molecular weight of 36 kDa which is smaller than the active mature enzyme (46 kDa) [46]. This last step of activation is probably performed by other lysosomal cysteine proteases. When THP-1 cells were treated by L-leucyl-L-leucine methyl ester (Leu-Leu-OMe), which makes lysosomal membranes permeable, legumain and cathepsin B activities were completely abolished in cell lysates from the cells (personal communication: Eilen Tungland). Leakage in lysosomal membrane forced the containing proteases towards the pH neutral environments in cytosol where the enzymes are not active. Upon activation by PMA and differentiation towards macrophages, THP-1 cells has showen dramatic increase in legumain activity (50-fold) and mRNA expression (500-fold) [44]. A 32 kDa legumain band appeared by immunodetection which was suggested to be consistent with active legumain in THP-1 cells.

The antibody used against legumain was self-made by immunizing rabbits with a human legumain peptide sequence.

To characterize different legumain forms (pro- and active) in HEK 293 cells in this study, immunoblotting of legumain was performed. Immunoblots of legumain using a commercial antibody against legumain (Abcam) in HEK 293 cells revealed three strong bands of approximately 57, 45 (double band) and 29 kDa, respectively. For comparison, immunoblotting of legumain in HEK 293 cells has earlier shown three strong bands as well, but with somewhat different molecular weights [72]. The 29 kDa protein band is assumed to be the molecular weight of active legumain, while the other bands are believed to be inactive proforms in HEK 293 cells in this study. In the literature, however, higher molecular weights (34 kDa, 35 kDa and 36 kDa) have been shown to be responsible for legumain activity [9, 46, 72]. The molecular weight of purified active pig kidney legumain appeared to be 34 kDa [9], while 35 kDa in purified rat and mouse kidney extracts [43, 44]. In HEK 293 cells, the active legumain has previously been shown to be a 36 kDa protein [72].

Furthermore, legumain activity in different elution fractions of HEK 293, THP-1 and PC12 cell lysates from size exclusion chromatography were measured to determine the molecular weight of active legumain in these cells. The amount of cells harvested and lysed for this purpose differed between the cell lines studied. Surprisingly, legumain activity was eluted in different fractions, with molecular weights of approximately 37, 32 and 25 kDa in HEK 293, PC12 and THP-1 cells, respectively. A definite explanation of these different molecular weights of active legumain in different cell lines is not known. A possible explanation might be that these different molecular weights represent different active forms of legumain which are not described yet, all capable of cleaving the specific substrate Z-Ala-Ala-Asn-NHMec. Importantly in THP-1 cells, the low molecular weight (25 kDa) active legumain may represent a legumain form involved in inflammation and possible the pathophysiology of leukemia. This deserves further elucidation.

Human and pig legumain have been described to have four potential glycosylation sites [9]. Several of the plant legumains, as well, are also known to be glycosylated. N-glycosylated pig legumain (34 kDa) was converted to a lower molecular weight form (31 kDa) as a result of deglycosylation by N-glycosidase F [9, 73]. Different glycosylation reactions with various

sugar groups might be another possible explanation to the observed varied molecular weight of legumain on Western or size exclusion chromatography in different cells.

The obtained molecular weight of active legumain in HEK 293 cells after size exclusion chromatography (approximately 37 kDa) and immunoblotting (as assumed; approximately 29 kDa) were different. This may be due to the fact that protein transfer through polyacrylamide gels with different concentrations (here 12 %) result in different molecular weights. Despite being a well established method for decades, Western blotting is not the most precise and reproducible method for assuming molecular weights of proteins. Since the calculated molecular weight of active legumain in HEK 293 cell lysate fractions (approximately 37 kDa) was in close agreement with what is described in the literature (36 kDa) [72], it may represent the active form of legumain in HEK 293 cells. The assumed molecular weight of active legumain in THP-1 cell lysate fractions in this study (25 kDa) appeared to be consistent with what was reported previously in our laboratory [44].

6.3 E64 and CA074 inhibit legumain activity in THP-1 cells

In an attempt to inhibit legumain activity in the cell fractions, different inhibitors were tested. Cystatin M (in media from CST6-transfected HEK 293 cells) inhibited legumain activity in fractions from all the cell lines studied. This is in consistent with the published literature about cystatin M which is described as a potent inhibitor of legumain (Ki 0.0016 nM) [27, 47, 53, 56]. In HEK 293 and PC12 cell lysate fractions, legumain activity was suppressed almost completely, while surprisingly only partially in THP-1 cells. The cathepsin-inhibitors E64 and CA074, as expected, did not affect legumain activity in HEK 293 and PC12 cells, whereas unexpectedly legumain in THP-1 cells was greatly decreased by these inhibitors. Similar surprising finding had previously been observed in individual experiments with THP-1 cells and RAW 264.7 cells (rat macrophages) in our research group without further investigations (personal communication: Professors Harald T. Johansen and Rigmor Solberg). This observation is in conflict with the published literature, where E64 and CA074 are not shown to influence legumain activity [42, 47]. For the first time such an unusual and remarkable phenomenon is being observed for legumain. The actual explanation of this observation in THP-1 cells is not identified. One possible explanation is that there might be two asparaginyl proteases having almost the same molecular weight, both capable of cleaving the specific substrate Z-Ala-Ala-Asn-NHMec. Legumain is probably the cysteine

protease which is inhibited by cystatin M, and the other unknown legumain-like protease may not be affected by cystatin M, but by cathepsin-inhibitors.

N-glycosylation of asparagine residues has been shown to block attack by legumain at otherwise sensitive sites [36, 74]. In addition, N-glycosylation of the asparagine residue Asn^{62} in cystatin F has been assumed to contribute to the reduced activity against legumain by altering the shape of the legumain-interacting surface [75]. Given that legumain has several potential glycosylation sites [9], it is tempting to speculate that glycosylation is partly responsible for the strange inhibition profile for legumain in THP-1 cells. The attached sugar groups may modify the conformation of inhibition sites that makes a sufficient interaction difficult between inhibitors and the glycosylated sites of legumain.

In order to further examine the legumain activity fractions in THP-1 cells and to compare with a well characterized cysteine protease, cathepsin B activity was measured in the same fractions. The molecular weight of the highest cathepsin B activity fraction in THP-1 was 29 kDa and differed from that of highest legumain activity (25 kDa) in these cells. This confirmed that cathepsin B was not represented in the 25 kDa legumain fraction and the activity of this fraction was caused by legumain or by a legumain-like protease rather than cathepsin B. Similarly, cathepsin B activity was measured in cell lysate fractions of HEK 293 and PC12 cells. Table 5 summarizes the correlation between molecular weights of legumain and cathepsin B activities in HEK 293, THP-1 and PC12 cells and the corresponding cell lysate fractions in this study.

Table 5: Correlation between the molecular weights of legumain and cathepsin B in HEK 293, THP-1 and PC12 cells (and the corresponding cell lysate fractions).

Cell line	**Legumain molecular weight (fraction no.)**	**Cathepsin B molecular weight (fraction no.)**
HEK 293	37 kDa (15)	29 kDa (17)
THP-1	25 kDa (18)	29 kDa (17)
PC12	32 kDa (16)	32 kDa (16)

6.4 Interactions between legumain and cystatin M

As mentioned earlier, cystatins are potent endogenous inhibitors of lysosomal cysteine proteases [27, 47, 53, 56]. All functional cystatins are inhibitors of cysteine proteases of the papain (C1) family, and some also inhibit enzymes in the legumain (C13) family [14]. The secreted type 2 cystatins (C, M/E and F) have been shown to be powerful inhibitors of legumain activity, while cytoplasmatic type 1 cystatins (stefins) are not inhibitors of legumain [14, 56]. Very recently, it was reported that carboxy terminal extended phytocystatins (plant cystatins) are bifunctional inhibitors of papain and legumain [59]. Silencing cystatin M/E by a siRNA has been shown to increase both legumain- and cathepsin B/L-activities in the cells [23].

In this study, legumain activity was observed to be dramatically decreased in CST6-transfected HEK 293 cells and this decrease was strongly correlated to the potency of the total inhibitory activity in the conditioned cell media. For example, 104 % increase of total inhibitory activity in media resulted in a 94 % decrease of legumain activity. The same correlation but to a lower extent was observed in cell lysates of a number of CST6-transfected melanoma cell lines. These differences between HEK 293 and melanoma cells may due to the nature of these cells, as melanoma cells are cancerous and might have distraught balance between proteases and inhibitors among other things.

Taking in to account that cystatin M and legumain have different cellular locations, extracellular and (mainly) intracellular, respectively, this grade of interplay is surprising. Whether the secreted cystatin M on its way through the translational and transport cell machinery “meets” legumain and inhibits its activity is a possible situation which needs to be further explored. Another hypothesis might be the possibility that secreted cystatin M may have affinity to cell membranes and in this way appear in cell lysates. This hypothesis became more likely by the fact that stimulation of un-treated HEK 293 with cystatin M-conditioned media caused a moderate inhibition of legumain activity in these cells. Interestingly, decrease in legumain activity in these cells was, again, correlated to the potency of the total inhibitory activity in the conditioned media used. On the contrary, the other studied cell lines (THP-1 and PC12) treated with cystatin M-conditioned media showed almost no regulation of legumain activity, regardless potency of the total inhibitory activity in the conditioned media used.

As mentioned earlier, despite locating mainly in lysosomes, legumain has recently been shown to be located extracellularly in the tumour microenvironment, associated with matrix and cell surfaces [48]. That makes it likely to suggest another possible explanation of the observable facts that over-expression of or exogenously added cystatin M to HEK 293 cells reduced legumain activity. The sub-cellular localization of legumain is not fully elucidated and legumain may also exist on the cell surface other than in the tumour microenvironments.

6.5 Legumain and cystatin M in cancer

Legumain has been shown to be over-expressed in tumours and has been linked to promotion of migration and increased invasiveness [42]. Interestingly legumain over-expression in tumour cells was strongly correlated with increased migration and invasiveness *in vitro* and *in vivo*. Legumain has also been documented to activate pro-MMP2, and thus increase degradation of extracellular matrix [37]. Very recently, legumain mRNA expression has been shown to be elevated in bovine endometrial tissues during early pregnancy establishment [76]. Consequently, local proteolytic activity of legumain has been assumed to play an important role in placentation, by regulation of trophoblast (embryo outer layer) invasiveness and endometerial remodelling essential for proper placenta formation.

Recently, legumain has been reported to be over-expressed in tumour associated macrophages (TAMs) [49]. TAMs contribute to promotion and proliferation of tumour cells by secreting growth and proangiogenic factors as well as metalloproteinases. A full-length murine legumain-based DNA vaccine [49] and an oral legumain based minigene vaccine [50] against TAMs resulted in suppression of tumour growth, metastasis, and angiogenesis in tumours.

Correspondingly, silencing cystatin M in a metastatic oral cancer cell line increased proliferation, invasion and motility of the tumour cells [23]. Interestingly, the activity of legumain and cathepsin B and L were increased in the tumour cells. Contrary, over-expression of cystatin M in a metastatic breast cancer cell line reduced proliferation, invasion and motility of the tumours and thus cystatin M has been described as a tumour suppressor candidate [22]. Both cystatin M and E64 caused reduction in motility and invasiveness of the tumourigenic and metastatic breast cancer cells.

To investigate whether CST6-transfection of melanoma cell lines had any effect on migration or invasiveness of these cells, pTracer- and CST6-transfectd MM 11 and MM 69b cells has been applied to a matrigel (personal communication: postdoc. Jon Briggs; figure 19) which resembles the basal membrane around blood vessels. There was observed a 30-40 % decreased invasiveness in the CST6-transfected MM 11 cells compared to control (figure 19A), whereas the CST6-transfected MM 69b cells showed approximately 10 % reduction in invasiveness (figure 19B).

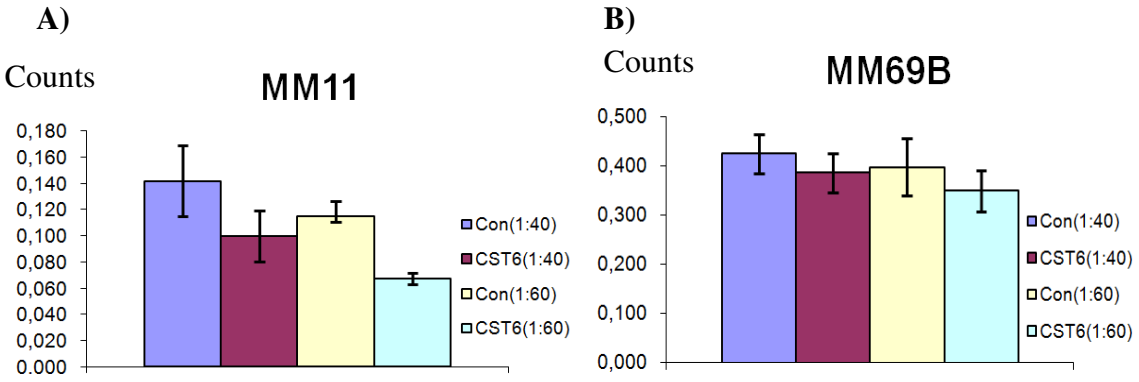

Figure 19 : Matrigel invasion analysis of the malignant cell lines MM 11 (A) and MM 69b (B) following CST6-transfection. *Melanoma cells were transfected with 4 µg CST6 or pTracer (control/ con). The following day the cells were counted and seeded equally into matrigel plates (matrigel dilution 1:40 or 1:60, respectively). Invasion into lower chamber was measured after 72 hours.*

Based on these observations and of legumain activity and inhibition characteristics in the same cells in this study, as well as involvements of legumain and cystatin M in cancer, it might be concluded that cystatin M is directly and greatly involved in reducing proteolytic activity of legumain. In conclusion, over-expression of cystatin M and thus inhibited activity of legumain results in decreased migration and invasiveness of tumour cells.

Recently, the gene for cystatin M (CST6) was reported to be frequently methylated and hence inactivated in a number of breast cancer cell lines when compared to normal breast-tissue samples [63]. This was shown to result in inhibition of transcription and silencing of cystatin M. Similarly, other reports have shown considerable reduction or loss of cystatin M expression in a number of skin cancer cell lines [27]. Premalignant and malignant breast cancer cell lines have been reported to express CST6 [27]. Expression of cystatin M in malignant cells was demonstrated to abolish several prominent functions such as cell proliferation, migration and matrigel invasion. For comparison, 10 of 12 established human breast cancer cell lines were shown to lack expression of cystatin M.

In an ongoing project of our research group in cooperation with RUH-HF, various melanoma cell lines have been screened for expressions of legumain and cystatin M [77]. It has been shown a small but not significant tendency of higher legumain activity in cell lysates and a corresponding lower total inhibitory activity against cysteine proteases in media from melanoma cells compared to non-melanoma cells. On the other hand, cathepsin B activity is shown to be significantly higher in melanoma compared to non-melanoma cell lysates and there is a positive correlation between cathepsin B activity and expression level. In addition, cystatin C has been observed to be more widely expressed in melanoma cells than cystatin M, and cystatin M has not been detected in the established melanoma cell lines. This is in agreement with the published literature in other cells [27].

7. Conclusions

The following findings were achieved in this study:

- A method for over-expression of cystatin M in HEK 293 cells was established.
- Cystatin M over-expression decreases legumain activity dramatically in the same cell and this is strongly correlated with the potency of total inhibitory activity in media.
- Treating living HEK 293 cells with exogenous cystatin M causes a moderate decrease in legumain activity, whereas legumain activity in living PC12 and THP-1 cells are not affected.
- Legumain in THP-1 lysate shows a previously unreported inhibitory profile, as its activity is directly decreased by E64 and CA074, respectively, and only partially by cystatin M.
- Legumain in HEK 293 and PC12 cells follows the known inhibitory profile as its activity is not affected by either E64 or CA074, but almost completely inhibited by cystatin M.
- The molecular weight of active legumain in HEK 293, THP-1 and PC12 cells was 37, 25 and 32 kDa, respectively.

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9. Appendix

9.1 Solutions

Cell culturing and harvesting

HEK 293 medium

Dulbecco's Modified Eagles Medium (DMEM) with 4500 mg/L glucose, 4 mM L-glutamine and 110 mg/L - sodium pyruvate	500 ml
FBS (heat inactivated, 56°C 30 min) (10 %)	50 ml
Penicillin (100 U/ml)-streptomycin (100 µg/ml)	5 ml

Autoclaved NaCl 0.9% solution

NaCl	9 g
dH_2O	1000 ml

Trypsin solution 0.5 %

Trypsin (10x) 5 %	5 ml
DMEM (without antibiotics and serum)	ad 50 ml

THP-1 medium:

RPMI 1640 medium w/2 mM glutamine and 1.5 g/l Na bicarbonate	500 ml
Glucose 4.5 g/l (10 %)	22.5 ml
1 M HEPES	5 ml
100 mM sodium pyruvate	5 ml
14.3 M 2-mercaptoethanol	2 µl
FBS (10 %)	50 ml
Penicillin (100 U/ml)-streptomycin (100 µg/ml)	5 ml
250 µg/ml Fungizone (amphotericin B)	5 ml

PC12 medium

DMEM (Gibco 42430-025)	425 ml
FBS (10 %)	50 ml
HS (5 %)	25 ml
100 mM sodium pyrovate	5 ml
Penicillin (100 U/ml)-streptomycin (100 µg/ml)	5 ml

PMA solution 40 ng/ml

PMA (40 nM)	40 µl
THP-1 medium	960 µl

Lysis buffer (pH 5.8)

$Na_3C_6H_5O_7.2H_2O$	100 mM
Na_2EDTA	1 mM
n-octyl-β-D-glucopyranoside	1 % (w/v)

Solution E

NaCl	8.5 g
KCl	500 mg
KHP_2PO_4	50 mg
Na_2HPO_4	60 mg
$MgSO_4.7H_2O$	200 mg
HEPES	5 g
$CaCl_2.2H_2O$	150 mg
dH_2O	ad 11

Tryptan blue (0.4 % w/v)

Tryptan blue	400 mg
Solution E	ad 100 ml

Solution is sterile filtered.

1 x PBS

$NaH_2PO_4.H_2O$	0.2 g
$Na_2HPO_4.H_2O$	1 g
$Na_3C_6H_5O_7.2H_2O$	6 g
NaCl	8 g
dH_2O	ad 11

***E. coli* culturing and transformation**

Autoclaved LB-medium

Tryptone	10g
Yeast extract	5g
NaCl	10g
Agar	15g
dH_2O	1 L

SOC medium

Tryptone	2 %
Yeast extract	0.5 %
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	20 mM

Agarose gel electrophoresis

5 x TBE, Tris-borate-EDTA

Tris	54 g
HBr	27.5 g
EDTA (0.5 M, pH 8.0)	20 ml
dH_2O	ad 1000 ml

1x TBE buffer solution

5x TBE buffer solution	200 ml
dH_2O	ad 1000 ml

Agarose gel (1%)

Agarose	1 g
1x TBE buffer solution	100 ml
Ethidium bromide (1 % in water)	5 µl

DNA-loading buffer

Bromophenol blue	0.25 g
Xylene cyanol FF	0.25 g
Ficoll	15 g
dH_2O	ad 100 ml

Protease activity measurements

Legumain activity measurements:

Legumain assay buffer (pH 5.8)

$C_6H_8O_7$ (citric acid)	39.5 mM
$Na_2 HPO_4$	121 mM
Na_2EDTA	1 mM
CHAPS	0.01 %

Legumain assay buffer with DTT

DTT (1 mM)	200 µl
Legumain assay buffer (pH 5.8)	40 ml

Z-Ala-Ala-Asn-NHMec in DMSO (2.5 mM)

Z-Ala-Ala-Asn-NHMec	13.3 g
DMSO	9.4 ml

Legumain substrate solution (34 µM)

Z-Ala-Ala-Asn-NHMec in DMSO (2.5 mM)	200 µl
Legumain assay buffer (pH 5.8)	14.5 ml

Cathepsin B activity measurements

Cathepsin B assay buffer (pH 5.5)

CH_3COOH 100 %	5.75 ml
CH_3COONa	200 mM
Na_2EDTA	4 mM

Cathepsin B assay buffer with DTT

DTT (200 mM)	400 µl
Cathepsin B assay buffer (pH 5.5)	9.6 ml
dH_2O	6.7 ml

Cathepsin B substrate solution (68 µM)

Z-Arg-Arg-NHMec in DMSO (1 mM)	680 µl
dH_2O	ad 10 ml

Total inhibitory activity measurements against papain

Papain assay buffer (pH 6.5)

$NaH_2PO_4.H_2O$	14 g
Na_2EDTA	0.4 g
Brij 35	0.1 g

Papain assay buffer with DTT (pH 6.5)

Papain assay buffer	30 ml
DTT (200 mM)	307 µl

Sodium acetate buffer (pH 4.5)

CH_3COOH (acetic acid) (1 M)	1.4 ml
dH_2O	ad 500 ml

Papain dilution (100 000X)

Dilution 1:

Papain suspension (18 mg protein/ml)	10 µl
Sodium acetate buffer (50 mM)	ad 1 ml

Final dilution (100 000X):

Dilution 1	10 µl
Sodium acetate buffer (50 mM)	ad 10 ml

Papain substrate solution (34 µM)

Z-phe-arg-NHMec in DMSO (1 mM)	680 µl
dH_2O	ad 20 ml

Total protein measurements

Bio-Rad protein assay colour solution in dH_2O (1:5)

Bio-Rad protein assay dye reagent concentrate	10 ml
dH_2O	40 ml

Western blotting

Gel buffer (1.5 M Tris-HCl pH 8.8)

Tris (base)	36 g
dH_2O	ad 200 ml

Stacking gel buffer (0.5 M Tris-HCl pH 6.8)

Tris (base)	3 g
dH_2O	ad 200 ml

SDS (10 %)

SDS	10 g
dH_2O	ad 100 ml

Ammonium persulphate (APS) 10 %

APS	5 g
dH_2O	ad 50 ml

Separation gel (12 %)

dH_2O	3.5 ml
1.5 M Tris-HCl pH 8.8	3.3 ml
Monomer stock solution	6 ml
SDS-solution (10 %)	130 µl
APS (10 %)	65 µl
TEMED	6.5 µl

Stacking gel (4 %)

dH_2O	6 ml
0.5 M Tris-HCl pH 6.8	2.5 ml
Monomer stock solution	1.3 ml
SDS-solution (10 %)	100 µl
APS (10 %)	50 µl
TEMED	10 µl

Electrophoresis buffer (pH 7.5)

Tris (base)	7.6 g
Glycine	36 g
SDS	2.5 g
dH_2O	2.5 L

Blotting buffer (pH 8.3)

Tris (base)	12 g
Glycine	58 g
Metanol	800 ml
dH_2O	ad 41

T-TBS (pH 7.5)

Tris (base)	2.5 g
NaCl	11.5 g
Tween 20	1 ml
dH_2O	ad 21

Blotto (Blocking solution)

Non-fat dry milk	25 g
T-TBS	500 ml

Analytical size exclusion chromatography

Gel filtration buffer (pH 5.0)

$Na_3C_6H_5O_7.2H_2O$	50 mM
NaCl	150 mM
Brij 35	0.01 %
β-mercapto ethanol	2 mM

Sterile filtered before use.

9.2 Protocols

Thawing, culturing and splitting HEK 293 cells

1. Take the Cryo-tube with the frozen HEK 293 cells out from the liquid N_2 tank and thaw it immediately at 37 ^oC.
2. Turn the tube constantly under thawing until a tiny lump of ice remains.
3. Transfer the cell suspension (1 ml) to a 75 cm^2 Corning flask and add 11 ml growth medium.
4. Incubate at 37 ^oC and 5 % CO_2 for 24 hour.
5. Aspirate old medium and add 12 ml fresh growth medium. Incubate (at 37 ^{o} C and 0.5 % CO_2) until splitting (80-90 % confluence, approximately every 4th day).
6. Aspirate medium and wash once in 10 ml sterile 0.9 % NaCl or DMEM without serum.
7. Add 2 ml trypsin-EDTA (0.5 % in DMEM without serum) to the 75 cm^3 flasks and let stay for 1 minute at room temperature. Bring the cells into suspension by tapping the side wall of the plate. Check in microscope.
8. Add 15 ml medium (with FBS to inactivate trypsin).
9. Transfer to a 15 ml tube and sentrifuge (800 rpm, 5 minutes).
10. Aspirate trypsin/medium
11. Resuspend in fresh medium (5-10 ml).
12. Count cells for experiments.
13. Seed the cells onto six-well plates and/or 75 cm^3 flasks as wanted.
14. Incubate at 37 ^{o} C and 0.5 % CO_2.

Thawing, culturing and splitting THP-1 cells

1. Take the Cryo-tube with the frozen THP-1 cells out from the liquid N_2 tank and thaw it immediately at 37 ^oC.
2. Turn the tube constantly under thawing until a tiny lump of ice remains.
3. Transfer the cell suspension (1 ml) to a 75 cm^2 Corning flask and add 9 ml growth medium.
4. Incubate at 37 ^oC and 5 % CO_2 for 24 hours.
5. Centrifuge the cells at 800 rpm for 5 minutes and aspirate medium.
6. Wash the cell pellet with RPMI without additives and resuspend in 8 ml RPMI medium.
7. Count the cells.

8. Transfer a volume corresponding to 1 million cells/ml (usually 1 ml) to a new 75 cm^2 Corning flask and add 9 ml growth medium and incubate for 72 hours at 37 ^oC and 5 % CO_2.
9. After 72 hours, new medium should be added (10 ml).
10. Split the cells whenever the concentration of the cells in the flask is about 1 million/ml (approximately once weekly) taking 1 million cells/ml and adding 9 ml RPMI medium.

Thawing, culturing and splitting PC12 cells

1. Add 10 ml medium in a 15 ml tube.
2. Put another 15 ml tube on ice.
3. Thaw cells at 37 °C until little ice left.
4. Transfer cells to the empty tube on ice.
5. Add medium over 2-3 minutes, resuspend.
6. Spinn cells (800 rpm, 5 minutes), resuspend in fresh medium.
7. Transfer to a culture flask.
8. Split (when 80-90 % confluence, approximately every 3-4th day): Aspirate old medium.
9. Hit flask to dislodge cells.
10. Add 5-10 ml fresh medium.
11. Break clusters by pipetting (x 5-10). Check in microscope.
12. Add 1 ml to a new flask containing 11 ml medium.
13. Remove old and add fresh medium every second day.

Cell freezing

HEK 293 cells

1. Treat cells with 2 ml trypsin-EDTA (to 75 cm^3 flasks) and let stay for 1 minute at room temp. Check in microscope.
2. Add 15 ml medium (with FBS to inactivate trypsin).
3. Transfer to a 15 ml tube and centrifuge (800 rpm, 5 minutes).
4. Aspirate trypsin/medium.
5. Make 10 % DMSO in DMEM (100 µl DMSO + 900 µl DMEM including FBS/antibiotics) on ice.
6. Resuspend cells in 10 % DMSO in DMEM (cold) to a final cell concentration of 2 millions/ml.
7. Transfer 1 ml per labelled Cryo-tube.
8. Set 30 minutes at -20 °C, then 2 h at -70 °C, before transferring to liquid N_2 for long term storage

THP-1 cells

1. Count cells and transfer to a 15 ml tube.
2. Centrifuge (800 rpm, 5 minutes).
3. Aspirate media
4. Make 5 % DMSO in media (0.5 ml DMSO + 9.5 ml growth media) on ice.
5. Resuspend in 5 % DMSO in growth media to a final volume of 1 million/ml.
6. Transfer 1 ml per labelled Cryo-tube.

7. Set 5-10 minutes on ice, 2 hours at -70 °C (dry ice), before transferring to liquid nitrogen for long term storage**.**

PC12 cells

1. Transfer cell suspension to a 15 ml tube and centrifuge (800 rpm, 5 minutes).
2. Resuspend cells in 1 ml cold medium (10 % FBS).
3. Make 20 % DMSO in FBS (200 µl DMSO + 800 µl FBS) on ice.
4. Add gently 20 % DMSO-solution to the cells, resuspend a few times (Pasteur) over 2-3 minutes on ice.
5. Transfer 1 ml per labelled Cryo-tube (approx 3-4 mill cells/tube).
6. Set 10 minutes on ice, 30 minutes at -70 °C (dry ice), before transferring to liquid nitrogen for long term storage**.**

Transformation of *E. coli* with plasmids

1. Thaw one 50 µl vial of One Shot® cells (chemically competent *E. coli*) (Invitrogen) on ice for 10 minutes.
2. Add plasmid DNA (1-2 µg), pipette gently to mix.
3. Let stay for 30 minutes on ice.
4. Incubate cells for 35 seconds at 42°C (heat shock).
5. Incubate cells on ice for 2 min.
6. Add 1 ml SOC-medium.
7. Incubate for 1 hour at 37°C on shaker (100 rpm).
8. Centrifuge the cells at the lowest possible rpm for 3 minutes.
9. Aspirate 900 µl of the supernatant, and re-suspend the cells using the remaining 100 µl of the supernatant.
10. Spread 100 µl onto an agar plate made with ampicillin.
11. Grow overnight at 37 °C.
12. Pick up only one bacterial colony from the agar plate and transfer to 3 ml liquid LB-medium and 3 µl ampicillin (50 mg/ml). Incubate at 37 °C under moderate agitation for 24 hours.
13. Prepare glycerol stocks in Cryo-tubes by gently mixing 800 µl bacterial suspension and 200 µl glycerol. Freeze at -70 °C.
14. Centrifuge the remaining cells (from stage 1) at 5000 g and 5 °C for 20 minutes and carefully remove all traces of medium. Then follow the protocol for QIAprep spin miniprep kit.

Isolation and purification of plasmids (Mini-prep)

QIAprep spin miniprep kit (QIAGEN):

1. Resuspend the pelleted bacterial cells in 250 µl Buffer P1 (kept at 4 °C) and transfer to an eppendorf tube. Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.
2. Add 250 µl Buffer P2 and mix gently by inverting the tube 4–6 times. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear, but do not allow the lysis reaction to proceed for more than 5 minutes.

3. Add 350 µl Buffer N3 and invert the tube immediately but gently and thoroughly 4–6 times. The solution should become cloudy.
4. Centrifuge for 10 minutes at 13 000 rpm (~17,900 x g) in a microcentrifuge. A compact white pellet will form.
5. Apply the supernatants from step 4 to the QIAprep spin.
6. Centrifuge at 13 000 rpm for 30–60 seconds. Discard the flow-through. Spinning for 60 seconds produces good results.
7. (Optional): Wash the QIAprep spin column by adding 0.5 ml Buffer PB and centrifuging for 30–60 seconds. Discard the flow-through.
8. Wash the QIAprep spin column by adding 0.75 ml Buffer PE and centrifuging at 13 000 rpm for 30–60 seconds. Spinning for 60 seconds produces good results.
9. Discard the flow-through, and centrifuge for an additional 1 minute to remove residual wash buffer.
10. Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris-HCl, pH 8.5) or water to the middle of each QIAprep spin column, let stand for 1 minute, and centrifuge for 1 minute.

Isolation and purification of plasmids (Maxi-prep)

Culturing of bacteria transformed with plasmid

1. From the *E. coli* stock in glycerol in the freezer (-70 ^{o}C), scrape a little amount of the bacterial stock by a scoop and seed onto an agar plate (with ampicillin) and let the bacteria grow overnight at 37 ^{o}C.
2. Pick up one bacterial colony and suspend in 3 ml liquid LB-medium, before adding 3 µl ampicillin (50 mg/ml). Incubate at 37 ^{o}C under moderate agitation for approximately 5 hours.
3. Transfer 100 µl of the bacterial suspension to a larger container with 180 ml LB-medium before adding 180 µl ampicillin (50 mg/ml). Incubate at 37 ^{o}C under agitation overnight.
4. Transfer to a centrifuge tube and follow the JETSTAR maxiprep protocol for plasmid purification from *E. coli*.

JETSTAR plasmid kit (maxiprep)

1. Centrifuge the bacterial suspension at 5000 g and 5 ^{o}C for 20 minutes and remove carefully all traces of medium.
2. Reconstitute the bacterial pellet by adding 10 ml solution E1. Continue until a homogenous suspension is formed.
3. Cell lysis occurs upon the addition of 10 ml solution E2. Mix gently by inverting until a homogenous cell lyate is formed.
4. Add 10 ml solution E3, in order to neutralize the lysate, mix immediately by multiple inverting until homogeneity is achieved. Transfer the mixture to a smaller centrifuge tube and centrifuge the mixture at 12000 g and 4 ^{o}C for 10 minutes.
5. While centrifugation, equilibrate the maxiprep column by applying 30 ml solution E4 to the column. Allow the column to empty by gravity flow.
6. The supernatant from step 4 is then applied to the column and allowed to run by gravity flow.

7. Wash the column once by adding 60 ml solution E5 and allow the column to empty by gravity flow.
8. Elute the plasmid using 15 ml solution E6. COLLECT this solution!
9. Precipitate the DNA with 10.5 ml isopropanol and centrifuge at 4 ^{o}C and 12000 g for 30 minutes. Wash the plasmid with 10 ml 70 % ethanol and air-dry the pellet for 15-30 minutes. The dried pellet is then re-dissolved in 500 µl of a suitable buffer (i.e. 10 mM Tris-HCl pH 8, TE buffer or sterile water). Transfer the plasmid solution to a microcentrifuge tube and store at 4 ^{o}C.

Agarose gel electrophoresis

1. Prepare a 1 % agarose gel by adding 100 ml buffer (1x TBE) to 1 g agarose.
2. Boil the solution in a microwave oven in order to dissolve the agarose (avoid superheating of the gel).
3. Add 5 µl ethidium bromide (1 %) while the gel solution is still warm.
4. Place a comb and pour the warm agarose (approximately 50 ^{o}C) into a form and let the gel stiffen at room temperature for 40 minutes.
5. Remove the comb before the addition of a sufficient amount of 1x TBE.
6. Load the samples into the wells.
7. Apply the electrical current (100 V for 1-2 hours).
8. Examine the gel by UV-light and take a photo.

Transient transfection of HEK 293 cells with LGMN- and CST6-plasmids

1. Seed 0.5 x10^6 cells/well one day before transfection (or 2.5-3 x10^6 cells/well three days prior to transfection) onto six-well plates. Up to 1 or 2 ml growth medium/well (for LGMN*- and CST6- transfection, respectively).
2. Prepare the following solutions for the transfection process per well:
   A) Plasmids (1-20 µg/ml) diluted in DMEM +/- serum and antibiotics (for CST6: only DMEM -/- serum and antibiotics).
   B) Lipofectamin 2000 (5-20 µl/ml) diluted in DMEM +/- serum and antibiotics (for CST6: only DMEM -/- serum and antibiotics).
3. Combine solutions A and B and mix gently. Incubate at room temperature for 30 minutes.
4. Remove media from the wells and overlay 1-2 ml Lipid-DNA solution (A+B) to the cells.
5. Incubate for 24-48 hours.

*Check table 2 for adjustments.

Measuring proteolytic activity of legumain and cathepsin B

1. Add 20 µl of samples or blank (lysis buffer) to a black 96-well microplate, each in triplicate. Temperature should be adjusted at 30 ^{o}C on the Wallac Victor.
2. <u>For legumain:</u>
   A) Prepare a solution containing 100 µl DTT (200 mM) and 20 ml legumain assay buffer. This solution should be freshly made before every assay.

B) Prepare legumain substrate solution by combining 200 µl Z-Ala-Ala-Asn-NHMec (2.5 mM in DMSO) and 14.5 ml legumain assay buffer.

For cathepsin B:

A) Prepare a solution containing 400 µl DTT (200 mM) and 10 ml cathepsin B assay buffer. Aspirate 1 ml and add 6 ml dH_2O. This solution should be freshly made before every assay.

B) Prepare cathepsin B substrate solution by combining 680 µl Z-Arg-Arg-NHMec (1.0 mM in DMSO) and 10 ml dH_2O.

3. Insert the buffer and substrate tubes in the Wallac Victor's injector positions 1 and 2, respectively.
4. On Victor, click/mark for the injectors, fill and apply respectively.
5. From the workout command, chose the proper protocol for the assay.
6. Place the 96-wells microplate in the instrument and start the assay.
   A) Each well will be added 100 µl assay buffer.
   B) Immediately before measurements, each well will be added 50 µl substrate solution.
7. Kinetic will be measured in ΔF/s.

Measuring total inhibitory activity against papain

1. Boil collected media samples (100 ^{o}C, 5 minutes) and centrifuge at 10000 g and 4 ^{o}C for 5 minutes. Transfer the supernatants to new eppendorf tubes.
2. Make different dilutions of the samples using serum-free medium. For example 80 %, 70 %, 60 %, 50 % etc.
3. Prepare following solutions:
   A) Papain solution: in an eppendorf tube, mix 10 µl of a papain suspension (18 mg protein/ml) and 990 µl sodium acetate buffer (1000 X), and transfer 10 µl to a tube containing 9990 µl sodium acetate (100 000 X).
   B) Papain peptide substrate solution (34 µM): Mix 680 µl Z-phenyl-arginin-NHMec and distillated water up to 20 ml.
   C) Activated papain assay buffer: Add 307 µl of 200 mM DTT to 30 ml papain assay buffer.
4. To the first 3 wells (blank) add 20 µl sodium acetate buffer and 17 µl papain assay buffer (without DTT).
5. To the papain control wells (usually 6 wells) add 20 µl papain solution and 17 µl serum-free medium.
6. To sample wells, add 20 µl papain solution and 17 µl of the different prepared dilutions of the media samples.
7. Chose the right protocol (from the software; workout) for the measurement of inhibitory activity and make adjustments if necessary. Keep the temperature at 37 ^{o}C.
   A) Every well (not the blanks) is added 83 µl of activated papain assay buffer (by the auto-injector), and the plate is then shaken in 10 seconds and incubated for 10 minutes at 37 ^{o}C.
   B) The wells are added 50 µl of the peptide substrate solution (34 µM Z-phenyl-arginin-NHMec) to a final concentration of 10 µM and the final volume is 170 µl.
8. The fluorescence is immediately measured and repeated 10 times with an interval of 1 minute and a shaking period of 3 seconds in-between each measurement.

Fluorescence measurements are performed at 355 nm excitation and 460 nm emission.

Measuring total protein concentration (standard version):

1. Prepare the colour reagent by mixing 1 part of Bio-Rad Protein Assay Dye Reagent Concentrate with 4 parts of distillated water. Sterile filtrate (0.2 µm) the solution.
2. To a transparent 96-well microplate (Costar), add 10 µl of standard solutions of serum albumin (0, 50, 100, 150, 200, 250, 300 µg/ml), which is diluted in lysis buffer, in parallels.
3. Add 10 µl of the samples to the microplate in parallels as well.
4. Apply 200 µl of the prepared colour reagent to each well.
5. Incubate the plate at room temperature for 5 minutes, before measuring the absorbance (at 595 nm) in the Wallac Victor.

Western blotting

A) Preparations:
Make T-TBS and the blocking solutions as described earlier.

B) Sample preparation (TCA precipitation): *(The samples are kept on ice during the whole process)*
   1. Add 1.2 ml sample to an eppendorf tube.
   2. Add 120 µl TCA (100 %) and mix well (vortex). Incubate on ice for 30 minutes and centrifuge at 10000 g and 4 ^{o}C for 5 minutes. Remove the supernatant.
   3. Resuspend the pellet with 1.2 ml TCA (1 %) and mix well by vortexing (can be difficult to resuspend). Centrifuge at 10000 g and 4 ^{o}C for 5 minutes and remove the supernatant.
   4. Add 30 µl 0.1 M NaOH and mix well (vortex).
   5. Add 10 µl 4X sample-buffer.
   6. Add 10 % β-mercapto ethanol and 5 % bromophenol blue calculated as percent of the final volume of the samples in order to have the same concentration of proteins in the samples.
   7. Add 1X sample-buffer. The amount is estimated based on the final volume of the samples.
   8. Boil the samples (100 ^{o}C) for 5 minutes.

C) Polyacrylamide gels:
   1. Put together different pieces of gel pouring equipment and make sure that the system is tight.
   2. Prepare the 12 % separation gel as described under solutions. APS and TEMED are added at the end.
   3. Mix well, pour the gel and add isobutanol (200 µl) to straighten the surface of the gel and remove air bubbles.
   4. Let stiffen for approximately 45 minutes.
   5. Remove isobutanol and wash with dH_2O.
   6. Make the 4 % stacking gel as described under solutions. Pour it on the separation gel and place a comb. Add more stacking gel solution until the form is filled.
   7. Let the gel stiffen for approximately 45 minutes.

8. Separate the gel pouring form carefully from each other and wash with dH_2O. Use the gel directly or pack in wet papers and keep at 4 ^oC until analysis.

D) Gel electrophoresis:
1. Set up the gel in an electrophoresis tank and fill with electrophoresis buffer (pH 7.5).
2. Apply samples (10-20 µg) and precision standard (2.5 µl) to the gel.
3. Connect water (to cool down the system) and start electrophoresis at 200 V for approximately 1 hour and 20 minutes or stop when bands reach the lowest part of the gel.

E) Membrane transfer (Western blotting):
1. Cut a nitrocellulose membrane and filter papers in suitable sizes and soak in blotting buffer (pH 8.3) for 10 minutes.
2. Prepare a sandwich from gels, membranes and filter papers on the blotting apparatus with the following layers in order from cathode (-) to anode (+): 1) filter paper (3 sheets) soaked in transfer buffer, 2) gel, 3) membrane, 4) filter paper (3 sheets) soaked in blotting buffer, 5) Mylar mask which points the electrical current towards the sandwich only. Add blotting buffer between each layer.
3. Subject the sandwich to an electrical current (64 mA for two gels and 32 mA for one gel) for 45-60 minutes.
4. Membranes are coloured in Ponceau S-solution for 1 minute, wash and make a copy.

F) Addition of antibodies
1. Put membranes in dH_2O for few minutes, followed by 10 minutes in T-TBS.
2. Block membranes with blocking solution (Blotto) for 60 minutes at room temperature.
3. Remove Blotto and add primary antibody (in Blotto). Ten ml Blotto is used for each membrane. Dilution of primary antibody depends on antibody type.
4. Incubate at 4 ^oC overnight under slight agitation.
5. Wash membranes with Blotto (3 x 10 minutes), under slight agitation, at room temperature.
6. Add secondary antibody (-HRP) in Blotto (10 ml). Dilution of secondary antibody depends on type of antibody.
7. Remove secondary antibody and wash with Blotto (3 x 10 minutes), under slight agitation, at room temperature.
8. Wash membranes with T-TBS (2-3 x 10 minutes), under slight agitation, at room temperature.

G) Evoking immunoreactive bands
1. Incubate membranes in enhanced chemiluminescense (ECL) solution (Amersham) for 1 minute. Mix reagents 1:1 (as recommended by the manufacturer).
2. Pack membranes in a plastic transparent film.
3. Turn off the light and put membranes together with an x-ray film in a cartridge.
4. Evoke immunoreactive bands (for 5, 15 and 60 minutes).
5. Incubate in a developer solution for 30 seconds, wash in water and incubate in a fixer solution for 5 minutes. Let films air-dry.
6. Quantify bands by the software GeneTools (Syngene).